ACTA PHYSIOLOGICA SCANDINAVICA

Fasc 1 (January 1975)

Short Communications

Fasc 2 (February 1975)

Short Communication

Fasc 3 (March 1975)

Fasc 4 (April 1975)

Short Communications

Supplements

INDEX AUCTORUM

Acta physiol scand 1975 93 1-9
From the Department of Physiology University of Goteborg Sweden

The Hypothalamus and Vagally Mediated Gastric Relaxation

By

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Received 8 January 1974

Abstract

LISANDER, B *The hypothalamus and vagally mediated gastric relaxation* Acta physiol scand 1975 *93* 1-9

Experiments with recording of gastric volume were performed in chloralosed cats Topical hypothalamic stimulations produced vagally mediated increases in volume by two mechanisms One type of response due to central inhibition of vagal excitatory tone was induced from the defence area and eliminated by atropine or vagotomy The other type of response far less commonly encountered remained after atropine and spinal cord section but was abolished by vagotomy The latter type was not induced from any well defined hypothalamic region and had a high stimulation threshold While sham feeding in conscious cats with esophagostomy and gastrostomy induced prompt marked and longlasting gastric volume increases in connection with swallowing, such responses were not evoked as anticipatory reaction to food intake The relaxations could be prevented by vagotomy but not by guanethidine nor atropine It is concluded that the vagally mediated relaxation in connection with sham feeding occurs mainly from activation of mechanoreceptors and that the hypothalamic control over the vagal relaxatory fibres is probably minor

The occurrence of gastric relaxation in connection with swallowing has long been known (Cannon and Lieb 1911) As shown by Martinson (1965) the stomach is supplied by two types of efferent vagal nerve fibre groups One group mediates a gastric contraction which is blocked by atropine the other group elicits a profund relaxation which is resistant to atropine and antiadrenergic drugs but blocked by ganglionic blocking agents (Martinson 1965) The latter relaxatory nerve fibres are the efferent pathway in vago-vagal reflexes (Jansson 1969 a Ohga *et al* 1969) which can be activated *e g* by graded esophageal distension (Abrahamsson and Jansson 1969) In the latter study it was also found that swallowing movements elicited by pharyngeal stimulation in the anesthetized cat produce a volume increase of the stomach through a reflex activation of the vagal relaxatory fibres

According to Abrahamsson (1973) the vago-vagal relaxatory reflex is present in decerebrated cats The question arises whether the vagal relaxatory fibre are influenced also from higher levels in the central nervous system and what influences apart from purely mechanical stimulation of the upper digestive tract may cause a reflex receptive relaxation of the stomach

It is well established that the lateral hypothalamic area integrates the control of food

intake and forms the feeding centre Folkow and Rubinstein (1965) noted that stimulation in this area induced eating in cats Identical stimulation with the animals under anesthesia caused increased motility and blood flow in the gastrointestinal tract It was suggested that this autonomically mediated feeding response takes place as an anticipatory response in an animal approaching food From preliminary findings it was concluded that this response pattern includes an activation of the excitatory as well as the relaxatory vagal fibres to the stomach This would result in an anticipatory volume increase of the stomach together with increased motility

In the present study the hypothesis of *hypothalamically controlled anticipatory gastric relaxation* was evaluated experimentally in two different ways In one group of experiments topical hypothalamic stimulations were performed in lightly anesthetized cats It was also investigated whether there is any activation of the vagal relaxatory fibres to the stomach in conscious cats allowed to see smell and taste food without ingesting it

Methods

Hypothalamic stimulation experiments

39 cats weighing 1 9–4 4 kg were used The animals were deprived of food 24 36 hours before the experiment After induction with ether they were anesthetized with chloralose 35–40 mg/kg b wt i v

The head of the animal was immobilized in a Horsley Clarke apparatus A monopolar stainless steel electrode was stereotaxically inserted into the hypothalamus A Grass S5 stimulator was used for delivering rectangular pulse waves of 1 2 ms duration and 2–10 V amplitude The frequency was varied within wide limits (20 100 Hz) In later parts of the study a constant current device was used delivering pulses of 0 1 0 5 mA In some experiments the spinal cord was transected between C3 and C4 or C6 and C7 and the animal was kept on artificial respiration To identify the stimulation points a direct anodal current of 1 mA was passed through the electrode for 30 s at the end of the experiment The brain was then perfused with 10 per cent formalin The hypothalamus was embedded in paraffin or celloidin after which serial sections 50 μ thick were cut and stained with the luxol fast blue method All electrode positions given in the present paper refer to the atlas by Jasper and Ajmone Marsan (1961) — In som experiments of this series the right vagal nerve was cut and the central end was stimulated to el cit reflex vagal relaxation (*cf* Jansson 1969 a) Stimulation parameters were 1 10 Hz 1–4 ms and 4–15 V

Gastric volume changes were re orded at low intragastric pressure (4 8 cm H_2O) In the first experiments isolation of the gastric lumen by ligatures around the esophagus and the pylorus was used (for deta ls see Jansson and Martinson 1965) However abdominal surgery apparently disturbed the conditions for recording of gastric motility when the extrinsic nerves were intact because of reflex activation of adrenergic (Jansson 1969 b) and vagal nonadrenergic fibres (Abrahamsson to be published) to the stomach Therefore in later experiments abdominal surgery was avoided and the gastric volume was recorded with a large rubber balloon introduced through the esophagus (for details see Jansson 1969 a)

Blood pressure was recorded by a mercury manometer connected to a catheter placed in a f moral artery Atropine (atropine sulphate Merck) was given i v in a dose of 0 2 0 5 mg/kg b wt while guanethidine (Ismelin® CIBA) was given in a dose of 4 mg/kg In some experiments gallamine triethiodide (Flaxedil® May & Baker) 4 mg/kg was used Artificial respiration was then maintained by a respiration pump

Chronic animals

This part of the study was carried out on ten cats of both sexes weighing between 3 and 4 kg All operative procedures were performed under Nembutal® (Abbot) 35 mg/kg i p with aseptic precaution In on operation the esophagus was externalized in the neck (Olbe 1959) and at a second stage 3 4 weeks later this part of the esophagus was provided with a cannula In addition a small abdominal incisi n wa made on the left side and a 3–4 cm long opening was made in the stomach slightly anterior to the greater curvature A wide bore teflon cannula (outer diameter ?4 mm inner diameter 18 mm) cor structed as

described by Thomas (1941) was placed in the opening of the stomach which was then narrowed by a purse string suture In some experiments the vagi were identified below the diaphragm and thin stainless steel wires were placed under the nerves The ends of the wires were drawn out through the abdominal wall allowing for vagotomy by pulling the wires

Postoperatively the animals received saline and isotonic glucose solution subcutaneously for up to 3 days and later milk gruel and minced fish and meat *ad libitum* In one animal the externalized esophagus was resected This animal was fed through the gastrostomy and fluids were given by stomach tube *via* the opening in the neck The sham feeding experiments were carried out at least two weeks after the operations when the cats were in good condition

The cats were as a rule starved for 36 h before the experiments In some cases the animals were fed the night before the recording and the next day the stomach was emptied through the gastrostomy and thoroughly rinsed with saline Gastric volume was recorded at constant intraluminal pressure by a large rubber balloon (approximately 10 cm in diameter) introduced through the gastrostomy and connected to a large volume reservoir The reservo r in turn was connected to a piston recorder writing on a smoked paper (for details see Jansson 1969 a) The intraluminal pressure was measured with reference to the surface of the table During the recording the cat was kept in a cage 15 cm high 15 cm wide and 50 cm long. The cats were usually sham fed for three min due to reconsumption it was sometimes d fficult to interrupt the feed ng at an exact moment Milk raw and boiled fish and m nced meat were used After the experi ments the stomach and the balloon were inspected for traces of food and if any were found the experi ment was d scarded In the course of the experiments atropine 0 2 0 55 mg/kg and guanethidine 2–4 mg/kg were injected i p Finally the animals were subjected to terminal experiments under chloralose anesthesia 50 mg/kg and the vagus nerves were electr cally stimulated in the efferent or afferent direction at 1–10 Hz, 1–4 ms and 4–15 V

Results

1 *Activation of vagal non adrenergic relaxatory fibres by hypothalamic stimulation*

Topical electrical stimulations were performed in the hypothalamus to activate the vagal high threshold non adrenergic relaxatory fibres In four of eleven atropinized spinal cord transected cats it was possible to induce a clear relaxation of the stomach Gastric volume was then recorded with the balloon method The stimulation points were A 14 L 1 H −4 A 12 L 1 H −3 A 12 L 1 5 H −3 and A 10 L 3 H −3 respectively Fig 1 illustrates a representative long lasting relaxatory gastric response The topically induced gastric relaxatory responses were however in all cases smaller than the volume increases obtained on reflex activation of the non adrenergic vagal fibres (*cf* Jansson 1969 a) In these experi ments as well as in cats with intact spinal cords it was difficult to obtain reproducible atropine resistant relaxations on repeated stimulations Stimulation thresholds were decidedly higher than those for eliciting inhibition of vagal excitatory activity (see later) The intensities used were 7–10 V at 1–2 ms duration and maximal responses appeared to occur at 50–100 Hz. In 2 cats bilateral vagotomy was performed and after this th relaxations could no longer be elicited

In 22 expts the hypothalamus was stimulated in atropinized cats with intact spinal cord In 15 of these the stomach was isolated by ligature around the esophagus and the pyloru The poor reproducibility of the vagally mediated responses rendered the interpretation of the effects of vagotomy and guanethidine administration difficult

2 *Inhibition of a vagal cholinergic influence to hypothalamic stimulation*

When gastric motor function is recorded with the balloon volume method the stomach is mostly under the influence of vagal cholinergic nervous activity (Jansson 1969 b) This was

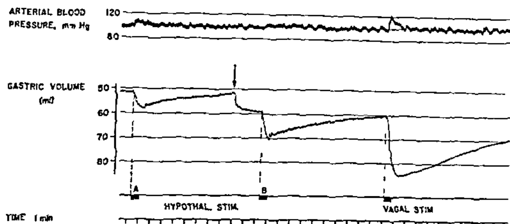


Fig 1 Cat 3 4 kg *Atropinized (0 5 mg/kg) Spinal cord transected and animal unilaterally agotomized* Relaxatory responses of the stomach to hypothalamic stimulation with 80 Hz 1 ms 10 V Stimulation point A 10 L 3 H −3 Note the similarity between these responses and the vago-vagal relaxatory reflex response elicited by electric stimulation of the central end of the cut right cervical main trunk (10 Hz, 2 ms 4 V) At the arrow an increase in intragastric pressure from 4 to 5 cm H O was made

the case also in animals with transected spinal cords (Fig 2 and 3) In eight spinal cats hypothalamic stimulations were performed before the administration of atropine and in all experiments gastric widening was induced (see Fig 2 and 3) In the experiments illustrated *in Fig 2 and 3 a prompt inhibitory gastric response (within 4 s) is recorded upon stimula* tion This type of response remained after guanethidine (given in 2 cats) but following atropine *hypothalamic stimulation failed to produce an inhibitory response despite the* fact that gastric tone could be decreased by a *reflex* activation of the vagal *relaxatory* fibres (Fig 2 Jansson 1969 a) Atropine without exception caused a fall in gastric tone in cats where this type of hypothalamically induced response could be elicited

Laparotomy was performed in two cats This procedure caused a fall in gastric ton and the hypothalamically induced gastric respons s could no longer b elicited Sub qu nt administration of atropine failed to influence stomach volum

Simultaneously with gastric motility heart rate and blood pressure were recorded *In* the experiment illustrated in Fig 3 the hypothalamic stimulation produc d an increase in heart rate together with the inhibitory gastric response Upon administration of atropine *there was an increase in resting heart rate (see Fig 3) The hypothalamic* stimulation also produced a pupillary dilatation *In all* eight exp rim nts *the re ponses were elicited from a* hypothalamic area corresponding to the defence area (Ab ahams Hilton and Zbrozyna 1960) at Horsley Clarke coordinates A 14–A 12 5 Pronounced responses could in all exp ri *ments be obtained with 1 ms 2–4 V (0 1–0 2 mA)* Optimal frequencies were b tween 60 100 Hz.

Chronic animals

Sham feeding caused gastric relaxation Fig 4 shows the reco ding of gastric *volume in a* cat with esophagostomy and gastrostomy The animal was sham fed for 3 min with large pieces of boiled fish The swallowing movements were followed by a prompt and pronounced

ARTERIAL BLOOD PRESSURE mm Hg
110
90
70
Atropine 0.5 mg/kg
VAGAL AFF STIM.
GASTRIC VOLUME (ml)
40
50
60
70
80
90
HYPOTHAL. STIM.
TIME 1 min

Fig. 2. Cat 3 1 kg Spinal cord transected *Inhibitory responses of the stomach to hypothalamic stimulation* (80 Hz, 2 ms 5 V) Stimulation point A 13 L 1 H −4 Note the complete disappearance of the inhibitory response after administration of atropine despite the fact that a pronounced vago vagal relaxatory response could be elicited Stimulation of the central end of the right cervical vagal nerve at 20 Hz, 3 ms 13 V Repeated doses of Flaxedil® were given i v

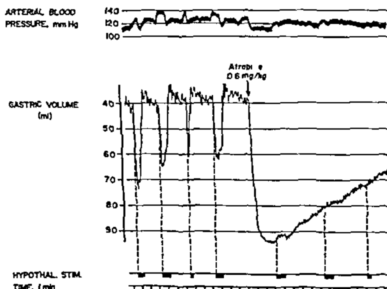


Fig 3 Cat 3 4 kg Spinal cord transected Inhibitory gastric responses on hypothalamic stimulation (80 Hz 1 ms 0 4 mA) at point A 14 L 1 H −4 Note the disappearance of hypothalamic responses after atropine Heart rate before atropine 140 beats/min During stimulation 168/min Heart rate after atropinization 160 min

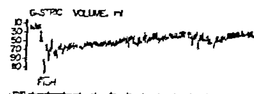


Fig. 4 Cat 3.3 kg. "Intragastric pressure" 11 cm H_2O Receptive relaxation with large pieces of fish.

increase of the gastric volume The first swallow preceeded the gastric relaxation for 5 s Minced meat or fish gave considerably smaller responses, indicating that the consistency of the food was of importance for the receptive relaxation Milk gave little or no effect, the only exception is included in Fig. 7 The responses were qualitatively similar regardless if the animal had starved for 36 h or the stomach had been emptied 2h to 20 min before the sham feeding. To exclude the possibility that the esophageal cannula caused an exaggerated stimulation of local receptors the externalized part of the esophagus was resected in one cat The gastric response on sham feeding was not clearly different in this animal.

These experiments suggest that the most important stimulus for receptive relaxation is the mechanical action of food in connection with swallowing. It was also investigated whether the sight and smell of food may be of importance Thus the cat was allowed to see food smell it and sometimes tiny amounts were placed on the nose so that the animal could taste it. Repeated swallows occurred under these conditions and the amount of saliva escaping though the esophagostomy was clearly increased The animal tried to reach the food, sometimes purred and showed all signs of good appetite However it was not possible to induce any receptive relaxation by other stimuli than swallowing. In two cats, however a shortlasting disappearance or decrease of the gastric motor waves was observed.

In Fig 5 the animal was prevented from reaching the food during the first 2 periods, A and B When the animal was allowed to eat and swallow (C) a marked receptive relaxation ensued even though the food passed through the esophageal fistula and did not enter the stomach

The gastric relaxations during sham feeding were qualitatively similar to those obtained on reflex activation of the vagal high threshold relexatory fibres in anesthetized cats (Fig. 1 *cf* Jansson 1969 a) or recorded on electrical stimulation of the distal ends of the cut vagal nerve (Fig. 7 lower panel *cf* Jansson and Martinson 1965)

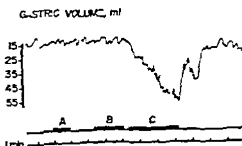


Fig. 5 Cat 3.5 kg "Intragastric press re 11 cm H_2O At A and B large pieces of fish were shown to the animal and tiny amounts were placed in the mouth No relaxation occurred. In C however a large volume increase was observed when the animal was allowed to eat

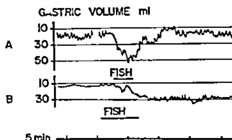


Fig 6 Cat 3 kg Intragastric pressure 11 cm H O The effect of sham feeding with large p eces of fish before and after atropine 0 5 mg kg i p Note that in panel A gastric contractions disappeared before animal was allowed to eat In panel B swallowing mo ements were considerably delayed

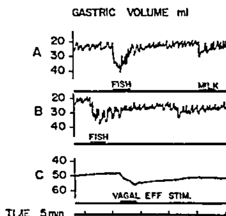
Three animals were given atropine The relaxatory responses remained after this drug (see Fig 6) Vagotomy prevented the relaxations completely in the 2 animals where this procedure was performed Guanethidine did not influence the rec ptive relaxations (2 cats see Fig 7)

Discussion

The main finding of the present study is the demonstration of two different vagally mediated gastric volume increase responses to hypothalamic stimulation

The first type can be elicited from different widely scattered points in the hypothalamus and any distinct functional zones for its eliciting could not be demonstrated This response was present in spinal animals It could be abolished by vagotomy but not by atropinization From this it can be concluded that the response is not dependent on sympathetic activation Further while mediated via the vagi it does not depend on cholinergic mechanisms Vagal fibres with these characteristics have been investigated by Martinson (1965)

The functional significance of this type of response to hypothalamic stimulation should be questioned Compared to vago-vagal reflexes the relaxations elicited were clearly of lesser magnitude and required also high stimulation intensity This appears to indicate that



F g. 7 Cat 3 3 kg Intragastric pressure 11 cm H_2O Sham feeding with large p eces of fish Note that in A the stomach relaxed upon sham feeding with milk Before B 4 mg kg of guanethid ne was gi en i p The an mal was anesthet zed before C and atrop ne (0 5 mg kg i v) was given The stomach pressure was set at 5 cm H O and both vagi were cut and stimu lated efferently at 4 Hz, ms and 7 V

either the hypothalamic control of these fibres is scant and functionally unimportant compared to the bulb r influence or the responsive hypothalamic neurons are so scattered that it is difficult to ectivate them by topical stimulation

A similar vagal noncholinergic gastric relaxation was elicited by Folkow and Rubinstein (1965) during stimulation of the hypothalamic feeding area These authors postulated that gastric relaxation is part of an anticipatory autonomic response to food intake To investigate whether there is any gastric anticipatory response to food intake experiments were carried out in unanesthetized cats with gastric and esophageal fistulas Also in these animals a non cholinergic vagal response could be elicited but the gastric relaxation was clearly not anticipatory under the present experimental conditions It failed in those animals who were only allowed to see smell and taste food Swallowing movements had to take place before the receptive relaxation occurred indicating that the response depended on receptors in the esophagus and/or the pharynx This assumption is further reinforced by the fact that the response greatly depended on the consistency and the type of food given to the animal

The second type of gastric vagally mediated relaxatory response to hypothalamic stimulation appears to be mediated via the cholinergic system Thus a background tonic vagal activity is needed to demonstrate the response Only those animals that responded to atropinization by a fall in gastric tone were the ones in whom the response to hypothalamic stimulation could be elicited Simultaneously the results of atropinization indicate that the response was of cholinergic nature Since the animals were spinalized it is clear that sympathetic mechanisms did not play a role in the mediation of this type of response to hypothalamic stimulation

This inhibition of vagal excitatory activity was elicited from a well defined region in the hypothalamus which corresponds anatomically to the defence area Thus it may be assumed that functionally this type of response is part of the general pattern of response to alerting stimuli known as the defence reaction However this could not be proven in the present experiments because the hallmark of the defence reaction the sympathetic cholinergic vasodilatation could not be demonstrated in the spinal animals That this gastric volume increase response may be part of a physiological adaptation such as the defence reaction is further supported by the fact that low stimulation intensity was sufficient for its demonstration Also this response was clearly induced in all the experimental animals further supporting the contention that it may have a physiological significance

The present experiments provide proof that there are two types of vagally mediated gastric volume increase responses and both may be induced from the hypothalamus Naturally the first type of response which is vagally mediated but not dependent on cholinergic mechanisms represents an interesting and less well studied facet of gastric motility control It is reasonable to assume that this type of response is predominantly bulbar and the hypothalamic modification of the response appears to be minimal On the other hand the second type of inhibitory response described is clearly under hypothalamic control and thus may appear under circumstances which are usually associated with hypothalamic control such as the defence reaction

This study was supported by grants from the Swedish Medical Research Council (14X-4 49 and 14P-4?70) and from the Faculty of Medicine University of Goteborg

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Acta physiol scand. 1975 93 10–14

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Disappearance of ^{3}H-Corticosterone from the Serum of Obese-Hyperglycemic Mice (Gene Symbol *ob*)

By

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Received 17 April 1974

Abstract

NAESER, P *Disappearance of ^{3}H-corticosterone from the serum of obese hyperglycemic mice (gene symbol ob)* Acta physiol scand 1975 *93* *10–14*

The disappearance of ^{3}H-corticosterone from the serum was measured in obese hyperglycemic mice and their lean litter mates of various ages. At 1 month of age the serum half life of the injected tracer was the same in the obese and lean animals. In the 2, 5 and 12 months old mice the tracer hormone disappeared faster in the obese mice than in their lean controls. The present results confirm that the previously observed enlargement of the adrenal cortex and the increased secretion of corticosteroids by adrenal glands *in vivo* in fact indicate a considerably hyperadrenocorticism in the obese mouse Furthermore the difference in disappearance rate of corticosteroids *in vivo* between obese and lean mice approximately parallels the age variation of the other metabolic abnormalities of the syndrome suggesting that the hyperadrenocorticism is part of the multiendocrine disturbance which manifests itself as the obese hyperglycemic syndrome

It was shown in a recent study that obese hyperglycemic mice have considerably higher concentrations of circulating corticosteroids than their lean litter mates (Naeser 1974) This observation agrees well with a previous report of an enlarged adrenal cortex in these animals (Hellerstrom *et al* 1962) and suggests a considerably raised secretory rate of corticosteroids. However attempts to evaluate the *in vivo* production and turn-over of adrenal cortical hormones in obese hyperglycemic mice are hampered by a lack of knowledge of the steroid elimination from the circulation In an attempt to solve this question the disappearance rate of ^{3}H-corticosterone from the serum of obese hyperglycemic mice was determined in the present study Since the manifestations of the obese hyperglycemic syndrome vary with respect to age the experiments were therefore performed in mice of different ages

Material and Methods

Altogether 35 male obese-hyperglycemic mice (genotype *obob*) and 31 male lean litter mates between 1 and 12 months of age were used. The mice belonged to a colony originating from The Jackson Laboratory Bar Harbor Maine USA and were reared at the Department of Histology University of Uppsala, Sweden since 19[illegible] as described previously (Naeser 1973)

All experiments were started between 8 and 10 a m 1β,-β ³H-corticosterone (specific activity 50 Ci/mmol The Radiochemical Centre Amersham England) was injected rapidly into the tail vein The radioactive corticosterone was diluted in 10 (v/v) alcohol to a concentration of 0 1 mCi/ml and given in a dose of 0 2 μCi/g b wt Between 4 and 14 animals were used in each group Blood samples were taken from the orbital vein plexus at 2 5 10 30 60 1 0 and 180 min after the injection

The blood was collected in dry test tubes and centrifuged after being allowed to clot at room temperature For determination of the radioactivity 10 μl serum was mixed with 7 ml scintillation fluid (Instagel Packard Instrument Company USA) and counted in a liquid scintillation spectrometer (Model 3380 Packard Instrument Company USA)

In order to confirm that the radioactivity of the serum collected at various times after the injection originated from H corticosterone 10 μl samples of serum were extracted with 500 μl dichloromethane (for spectroscopy E Merck AG Darmstadt W Germany) The dichloromethane fraction was evaporated and the residue redissolved in 50 / l chloroform The chloroform extract was then chromatographed on silical gel thin layer plates (TLC plates Silica Gel 60 E Merck AG Darmstadt W Germany) in a chloroform-ethyl acetate (1 50 v/v) ascending system (cf Quesenberry and Unger 1964) using unlabelled corticosterone as a reference standard The corticosterone spot was localized in UV light through its fluoroscence after being sprayed with a solution of 0 2 (w/v) 2'7 -dichlorofluorescein (E Merck AG Darmstadt, W Germany) in ethanol isolated and measured for radioactivity as described above The rest of the silica gel of the plate was also analyzed for presence of radioactivity

The calculations of the serum half life of the H-corticosterone tracer were based on the assumption that the volume of tracer distribut on would approximately fit the two-compartmental open system model as suggested by Riegelman et al (1968) The following formula

$$k = \frac{1}{\frac{A}{\alpha(A+B)} + \frac{B}{\beta(A+B)}}$$

was used to calculate the coefficient of disappearance where α is the slope of the first steep part (between 2 and 5 min) and β is the slope of the terminal linear part of the curve between 120–180 min A and B are the intercepts of the two respective parts of the curve on the Y-axis The results have been expressed as the mean values ± S E M The significance of differences between the various groups were tested by Students t test (Snedecor 1956)

Results

At all times after the injection of the radioactive tracer the position of the radioactivity in the thin layer chromatogram coincided with that of the marker corticosterone Radioactive counts obtained from the serum samples were considered therefore to be valid indicators of the concentration of the corticosterone in the serum and were used to calculate the half life of the hormone

The disappearance of the H-corticosterone from the serum in obese and lean mice of various ages is shown in Fig 1 The radioactive count of the serum sample obtained at 2 min after the injection was considered as the 100 % value and the radioactivity of the subsequent samples expressed as fractions thereof As can be seen in Fig 1 there was a rapid decline in the serum concentration of the tracer during the first 10 min period followed by a slower disappearance rate It is also seen that in all age groups the obese mice displayed a considerably faster elimination of the radioactive tracer from serum i e the curves were displaced downwards

The calculations of the half lives of H-corticosterone in serum from the various experimental groups are given in Table I the 1 month-old obese and lean animals displayed similar

costeroids increased possibly indicating a decreased production of corticosteroids *in vivo* Althogether these observations further support the view that an increased production of corticosteroids is an important factor in the obese hyperglycemic syndrome To what extent this hyperadrenocorticism reflects a disturbance of the feed back control of the pituitary adrenal axis remains to be settled

This work was supported by the Medical Faculty of the University of Uppsala The Swedish Diabetes Association and The Swedish Medical Research Council

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Acta physiol scand 1975 93 15–24
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Prostaglandin Release and Mechanical Performance in the Isolated Rabbit Heart during Induced Changes in the Internal Environment

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Received 8 May 1974

Abstract

WENNMALM Å *Prostaglandin release and mechanical performance in the isolated rabbit heart during induced changes in the internal environment* Acta physiol scand 1975 93 15–24

The isolated rabbit heart was perfused according to the Langendorff technique Prostaglandins in the effluent from the organ were identif ed by use of th n layer chromatography and assayed on the rat stomach strip The effect of alterat ons of the physical and chemical conditions of the perfusion medium on the overflow of prostaglandins from the heart was studied In addition the capacity of noradrenal ne and acetylcholine to release prostaglandins was tested Acidosis hyperthermia hypothermia hypotension hyperosmolarity and increased [K] or [Ca +] levels wh le all inducing marked changes in the mechanical activity of the heart did not induce prostagland n release Hypoxia on the other hand stimulated the liberation of prostaglandins Noradrenal ne was a potent agent for stimulat on of prostaglandin release in the absence of alpha and betaadren rgic receptor blockade Acetylcholine was also found to l berate prostaglandins by activation of muscarinic receptors The prostaglandin releasing capacity of acetyl choline was about 3 times weaker than that of noradrenal ne It is concluded that the release of prosta glandins from the rabbit heart is not dependent on the mechan cal activity of the organ Furthermore it is suggested that prostaglandins released by hypoxia may play an important role in th de elopment of reactive hyperem a Finally it is stated that the release of prostaglandins from the heart caused by acetyl chol ne may constitute the negati e link in an endogenous prostaglandin mediated feed back inhibition of the release of acetylcholine from parasympathet nerve endings

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Prostaglandin release from tissues can be evoked by different types of stimuli It has earlier been shown that sympathetic nerve stimulation as well as infusion of adrenaline causes release of prostaglandins from the dog spleen (Davies Horton and Withrington 1968 Gilmore Vane and Wyllie 1968) The observation has been confirmed in the vas deferens of the guinea pig (Swedin 1971) and in the rabbit heart (Samuelsson and Wennmalm 1971) Other vasoactive drugs reported to cause prostaglandin release are bradykinin (McGiff *et al* 1972) and angiotension I and II (Needleman *et al* 1973 McGiff *et al* 1970 a) Recently Minkes Douglas and Needleman (1973) observed prostaglandin release from the isolated rabbit heart following infusion of adenosine triphosphate or adenosin-diphosphate

Vibration has also been found to cause prostaglandin release from slices of rabbit spleen

(Gryglewski and Vane 1972) In the venous effluent from the cat or dog kidney prostaglandins have been reported to occur following renal ischemia (McGiff *et al* 1970 b Jaffe *et al* 1972)

The physiological significance of the prostaglandins liberated is still unclear in many respects It has been suggested (Hedqvist 1970) that prostaglandins released by sympathetic nerve stimulation modulate the response of the effector organ stimulated and this hypothesis has been supported by experiments where the endogenous synthesis of prostaglandins was inhibited in isolated organs (Samuelsson and Wennmalm 1971) as well as intact animals (Stjarne 1971 Junstad and Wennmalm 1972) Recently it has been suggested that endogenously formed prostaglandins mediate reactive (Kent *et al* 1973 Kilbom and Wennmalm 1974) hyperemia

Prostaglandins liberated from tissues might thus mediate or modulate important physiological mechanisms The further understanding of the physiological actions of endogenous prostaglandins seems to require a more systematic analysis of which types of stimuli that are capable to elicit prostaglandin release Therefore it was of interest to study if alterations in the internal environment possess the ability to initiate prostaglandin formation and liberation

Methods

a Rabbit hea t perfusion Rabbits of mixed strains and sexes were used for the study The weight of the animals varied from 1 ? to 2 4 kg After a blow on the head the animal was exsanguinated by cutting the left carotid artery The heart was rapidly remo ed and transferred to the perfusion apparatus where it was perfused according to Langendorff with Tyrode solution of the following compositio (in mM) NaCl 136 9 KCl 2 7 $CaCl_2$ 1 8 $MgCl_2$ 1 0 $NaHCO_3$ 11 9 NaH_2PO_4 0 4 glucose 5 6 The solution was continuously aerated with 5 CO_2 in O_2 The pH of the solution was 7 4 7 5 The perfusion pressure was 60 cm H_2O and the temperature was kept at 37 C The apex of the heart was connected to a strain gauge transducer and heart rate and contractile force were recorded on a Grass Model 5 D Polygraph The heart rate recording was repeatedly calibrated during the course of the experiments The strain gauge transducer was linear in the load range 0–5 ponds and thus permitted the qualitative recording of changes in contractile force during the experiments The experiments were started after the heart had been spontaneously beating in the perfusion apparatus for 10 15 min During the experim nts the effluent from the organ was collected in con ecutive 10 min periods for 40 min The effluent was immediately acidified to pH 3 and chilled

The perfusion apparatus (Dinkelacker & Co Mainz) consisted of two shanks 55 cm in height supplied with Tyrode solution from separate reservoirs In the shanks the solution was continuously heated and aerat d by separate thermostated circulation pump (Lauda Therm) and gas tube (AGA Stockholm) systems The shanks of the perfusion apparatus were joined together at the lower end with a stop-cock which allowed instantaneous changing of the perfusion from either of the shanks The system described thus permitted perfusion solutions of different composition reacti ity temperature and pressure to be kept in the two shanks The modified Tyrode solution the prostaglandin releasing capacity of wh ch was to be investigat d as regularly used for perfusion during the second perfusate collection peri d (min 10– 0) It was modified concerning its chemical composition so that the effect of acidosis and hyperosmolarity increased potassium or cal ium concentration and decreased glucose concentration or oxygen tension could be studied In other experiments the heart was perfused during the second perfusate c llection period at increased or decreased temperature or at a decreased perfusi n pressure

In some experiments acetylchol ne or noradrenaline were infus d through a cannula immediately abo e the aorta at a rate of 8 and 5 μg min respectively

The first (min 0 10) and the fourth (min 30–40) perfusat collection periods were used as control intervals and the prostagland n content of these effluents wa r garded as the basal The liberation of prostaglandins during these periods was compared to that observed dur ng the second (min 10 0) period

during which the experimental parameter to be studied (acidosis high temperature noradrenaline infusion etc) was maintained The third perfusate collection period in most experiments served as a recovery period and the effluent collected was discarded However in some cases (*cf* results) also the effluent during this period was analyzed for prostaglandins

b Anal s s of prostagland ns Lipids in the chilled and acidified effluent from the heart were extracted twice with equal amounts of ethyl acetate After washing of the organic phase to neutral reaction it was evaporated to dryness The residue was dissolved in 2 ml of water The biological activity of the dissol ed residue was tested against known amounts of PGE_2 on the superfused rat stomach strip (Vane 1957) The assay organ was superfused by Tyrode solution of the composition given above to which was added phentolamine (7 10^{-7} M) propranolol (8 10^{-} M) atropine (10 ^{7} M) methysergide (6 10^{-} M) and d phenhydramine (7 10^{-} M) in order to block activity in the strip due to the presence in the lipid extract of noradrenal ne acetylcholine serotonin and histamine

Usually 1/10 of the lipid extract from the effluent was tested on the stomach strip Since the assay organ was sensitive to prostaglandins of the E series in amount of less than 1 ng outflow rates of prostaglandin like substances in the effluent from the heart of less than 1 ng/min could be detected Each lipid extract was tested twice and the intra individual variation in the samples at repeated assays was usually less than 15 The recovery of the prostaglandin extraction procedure was checked by the use of internal standards, and ranged between 65 and 80 The values given for outflows of prostaglandins are not correct d for losses during the extraction procedure

For identif cat on of prostaglandins in the effluent from the heart thin layer chromatography (system AII Green and Samuelsson 1964) was used

c Stat t cal an lysis Values in the text and tables are given as mean±S E. For analysis of the increased outflow of prostaglandins during or following a 10 min period during which alterations in the perfusion conditions were appl ed the outflow of prostaglandins during this period was compared to the mean of th outflow observed during the preceding and the following 10 min period The differences were analysed using the paired t test

Results

The spontaneous beating rate of the hearts in the perfusion apparatus ranged between 105 and 165/min It usually declined somewhat during the course of the experiments as did the contractile force The coronary flow ranged between 25 and 45 ml/min at the beginning of the experiments and was also found to be moderately decreased towards the end of the perfusion The initial spontaneous outflow of prostaglandins in the perfusate ranged between 0 9 and 4 2 ng/min The outflow varied insignificantly between consecutive periods of effluent collection provided the heart was allowed to beat spontaneously In most experiments a weak tendency of the outflow of prostaglandins to decline during the course of the experiments was observed

a Physico-chemical interventions

Acidosis (perfusion at pH 7 1 during min 10–20) caused a slight decrease in heart rate from 146±8 to 133±8 beats/min The contractile force was moderately decreased by 30±7 % The coronary flow was increased from 34 to 46 ml/min The overflow of prostaglandins however was unaffected

Hyperthermia (perfusion at 40 C during min 10–20) increased the heart rate from 138±17 to 173±21 beats/min The contractile force decreased by 16±1 % and the coronary flow from 37 to 29 ml/min The outflow of prostaglandins displayed a weak and insignificant increase during the hyperthermia period (Table 1)

Hypothermia (perfusion at 23 C during min 10–20) decreased the heart rate markedly from 143±3 to 57±7 beats/min The contractile force was augmented by 56±20 No

change in the coronary flow rate or in the outflow of prostaglandins was observed during the period of hypothermia

During *hypotension* (perfusion at 30 cm H_2O during min 10–20) the heart rate was diminished from 107±2 to 82±7/min and the coronary flow from 36 to 11 ml/min The contractile force was slightly elevated by 12±6% The outflow of prostaglandins was unaffected

Perfusion at an *increased osmotic strength* (385 mOsm/l) did not affect the beating rat of the heart while the coronary flow rate as well as the contractile force were facilitated (from 33 to 45 ml/min and by 25±9% respectively) The overflow of prostaglandins in th effluent was unchang d during the p-riod of hyperosmolarity

Elevation of the potassium concentration of the perfusion solution (to 8 1 meq/l) did not affect the heart rate but decreased the contractile force by 28±4% The coronary flow was increas-d from 26 to 35 ml/min during the period of perfusion at a high [K] The PG outflow was unaffected

Increased calcium concentration (10 8 meq/l) induced a slight increase in heart rate from 145±6 to 160±6 beats/min and a marked increase in contractile force (46±7°) The coronary flow was insignificantly elevated (from 21 to 23 ml/min) The liberation of prostaglandins remained low at perfusion with increased concentration of calcium

b Metabolic interventions

Perfusion of the heart with a *hypoxic Tyrode solution* (5 % O corresponding to a pO_2 of 35 mm Hg) induced a marked coronary vasodilation as evidenced by an increas- in the flow rate from 29 to 45 ml/min The heart rate could not be recorded since ventricular arrythmia was induced by the hypoxia in most of the experiments The contractile force was markedly decreased by 75 to 100% (mean 81 ±4%) Following discontinuation of the hypoxia regular supraventricular rhythm was restored in most cases The contractile force also returned towards the prehypoxic value in most cases complete recovery was however not achieved (Fig 1) During hypoxia the liberation of prostaglandins was low Immediately following discontinuation of perfusion with low pO_2 a significant increase in the outflow of prostaglandins was observed (Table I) Thin layer chromatography revealed that the biological activity was due the presence of PGE mainly PGE_2 in the lipid extract of the effluent The outflow rate of prostaglandins remained elevated during the last perfusate collection period (min 30–40)

Absence of glucose did not significantly affect the mechanical performance of the heart the beating frequency remained unchanged and the contractile force displayed only a minor change (−6±8%) The coronary flow showed an elevation during the p riod of hypoglycemia (from 26 to 37 ml/min) The outflow of prostaglandins decreas-d continuously during the four perfusate collection periods in this series without any change during the hypoglycemia

c Transmitter infusion

Infusion of *noradrenaline* at a rate of 5 μg/min during 10 min caused a pronounced increase in heart rate (from 114±7 to 152±15 beats/min) and contractile force (63±14°) (Fig 2)

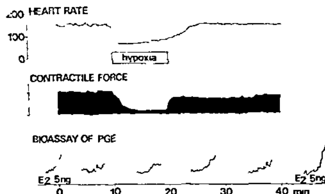


Fig 1 Perfused rabbit heart The upper two tracings show heart rate and contractile force at rest and during hypoxia (maintained during the time interval indicated by the box) The lower tracing demonstrates the contractions of the rat stomach strip when exposed to known amounts of PGE_2 or to 1/10 of the dissolved lipid extract of the effluent from the heart, collected in 10 min periods corresponding in time to the bioassay tracing

The coronary flow rate was likewise facilitated from 33 to 43 ml/min During the infusion of noradrenaline considerable amounts of prostaglandins appeared in the effluent from the heart (Table I) They were identified using thin layer chromatography as PGE_2 The outflow rate ranged from 7 4 to 15 3 ng/min The increase in prostaglandin overflow during the infusion of noradrenaline was 7 6 ng/min corresponding to 1 5 ng/μg noradrenaline infused Addition of alpha (phentolamine 3 10 M) and beta adrenergic (propranolol 4×10^{-6} M) blocking agents to the Tyrode solution completely abolished the mechanical response of the heart to the infusion of noradrenaline as well as the augmentation of the coronary flow caused by this drug In addition the increased overflow of prostaglandins caused by the noradrenaline infusion disappeared in the presence of adrenergic blocking agents

Infusion of *acetylcholine* at a rate of 8 μg/min decreased markedly the beating rate of the heart from 131 ±4/min to 51 ±10/min The contractile force was also diminished by 25 ± 3 (Fig 3) The coronary flow increased slightly from 36 to 40 ml/min The overflow of prostaglandins in the effluent during the infusion was augmented by 4 3 ng/min corresponding to 0 5 ng/μg acetylcholine infused The outflow of PGE from the heart caused by acetylcholine was chromatographically identified as both PGE and PGE Addition of atropine (10^{-6} M) to the medium perfusing the heart completely abolished not only the mechanical response to infusion of acetylcholine but also the outflow of prostaglandins caused by this drug

Discussion

The aim of the present study was to analyse the influence of variations in the internal environment on the formation and liberation of prostaglandins from the rabbit perfused heart The method used was to separately study the effect of different alterations in the perfusion conditions on the appearance of prostaglandins in the effluent from the organ It was also

TABLE I Prostaglandin outflow expressed in *ng/min* from the perfused rabbit heart during four consecutive 10 min periods. Outflow of prostaglandins significantly differing from the mean outflow during the preceding and the following period is indicated by = P<0 05 = P<0 01 * = P<0 001

Perfusion conditions	I 0–10	II 10–.0	III .0–30	IV 30–40
1 *Physico-chemical interventions*				
a acidosis				
pH during II = 7 1	3 0±1 0	3 4±0 7		2 0±0 6
pH during I III and IV = 7 4	(7)	(7)		(7)
b hypertermia				
perf temp during II = 40 C	0 9±0 4	1 4±0 3		1 1±0 5
during I III and IV = 37 C	(4)	(4)		(4)
c. hypotermia				
perf temp during II = 23 C	2 1±0 5	1 7±0 4		1 7±0 1
during I III and IV = 37 C	(4)	(4)		(4)
d hypotension				
perf pressure during II = 30 cm H_2O	1 1±0 7	0 5±0 3	0 4±0.	0
during I III and IV 60 cm H_2O	(3)	(3)	(3)	(1)
e hyperosmolarity				
osm. pressure during II = 385 mOsm/l	3 7±0 2	3 9±0 8		6±0 6
during I III and IV = 3 0 mOsm l	(7)	(7)		(7)
f hyperkalemia				
(K) during II = 8 1 mekv/l	1 1±0 ?	1 5±0 8		1 6±0 4
during I III and IV = 2.7 mekv/l	(3)	(3)		(3)
g. hypercalcemia				
(Ca) during II = 10 8 mekv/l	1 4±0.	1.2±0 3		1 1±0.
during I III and IV = 3 6 mekv/l	(4)	(4)		(4)
2. *Metabolic interventions*				
a hypoxia				
pO_2 during II 35 mm Hg	3 6±1 6	1 7±0 4	5 3±1 5	4±0 9
during I III and IV = 675 mm Hg	(5)	(5)	(5)	(4)
b low glucose				
(glucose) during II 0 mM/l	4 ±0 9	2 6±1 1	1 7±1 1	1 6
during I III and IV 5 6 mM/l	(4)	(4)	(4)	(1)
3 *Tra smitter infusion*				
a noradrenaline (50 μg)	.0±0 4	11 0±.1 *		4 8±1 7
infused during II	(4)	(4)		(4)
b noradrenaline (50 μg) infused during II phentolamine (3 10⁻⁶ M) and propranolol (4 10⁻⁶ M) present during I–IV	.1±0 4	6±0 2		1 8±0 5
	(3)	(3)		(3)
c acetylcholine (80 μg) infused	.0±0 3	6.±1 0		1 6±0 6
during II	(8)	(8)		(8)
d acetylcholine (80 μg) infused during II atropine (1 mg l) during I IV	1 1±0 1	1 8±0 8		1 9±0 9
	(4)	(4)		(4)

the aim of the study to make a comparison between the prostaglandin releasing capacity of the two neurotransmitters normally occurring in the heart acetylcholine and noradrenaline

Apart from the two neurotransmitters hypoxia was the only significant stimulus for prostaglandin release in the current study Various changes in the mechanical performance of the heart were induced during the experiments Thus, increased contractile force appeared parallel to an unaffected heart rate (hyperosmolarity) as well as to an increased (elevated

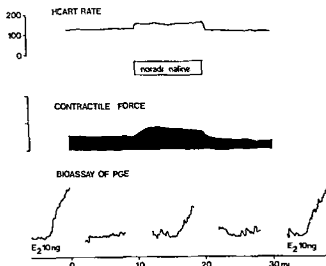


Fig 2 Perfused rabbit heart The upper two tracings show heart rate and contractile force at rest and during noradrenaline infusion (maintained during the time interval indicated by the box) The lower tracing demonstrates the contractions of the rat stomach strip when exposed to known amounts of PGE_2 or to 1/10 of the dissolved extract of the effluent from the heart collected in 10 min periods corresponding in time to the bioassay tracing

[Ca]) or a decreased (hypothermia hypotension) heart rate Despite these differences in the mechanical activity of the organ the prostaglandin liberation was constant A decrease in contractile force appeared at an unchanged heart rate (increased [K]) as well as at an increased (hyperthermia) or a decreased (acidosis) heart rate Neither during these perfusion conditions could any increase in the overflow of prostaglandins in the effluent be observed The observations clearly show that the mechanical performance *per se* is not decisive for the liberation of prostaglandins from the heart It has earlier been suggested that prostaglandin release from the dog spleen following sympathetic nerve stimulation or infusion of adrenaline is a consequence of the contractions of the organ in response to these stimuli (Gilmore Vane and Wyllie 1968) Results possibly pointing in the same direction have been obtained following mechanical vibration of chopped rabbit spleen slices where an increased prostaglandin formation was observed (Gryglewski and Vane 1972) However the hypothesis that increased mechanical activity is followed by an augmented prostaglandin formation is apparently not valid in the rabbit heart as evidenced from the current results Our results seem to be in contrast to those of Block and Vane (1973) who observed a decreased prostaglandin liberation from the rabbit heart during fibrillation However those authors reported a basal outflow of prostaglandins which was considerably higher than in the current series which might explain the difference

In the present study hypoxia was constantly found to cause liberation of prostaglandins from the heart Recent reports in the literature have displayed divergent results on this point Kent *et al* (1973) found that anoxia caused prostaglandin release from dog hearts and suggested that endogenous prostaglandins are important in the regulation of the coronary blood flow Block and Vane (1973 *cf* above) after induction of anoxia in the

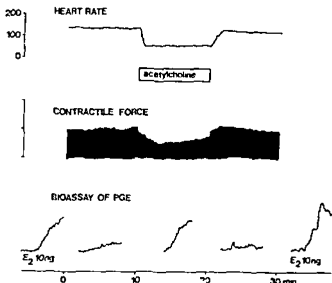


Fig 3 Perfused rabbit heart The upper two tracings show heart rate and contractile force at rest and during infusion of acetylcholine (maintained during the time interval indicated by the box) The lower tracing demonstrates the contractions of the rat stomach strip when exposed to known amounts of PGE_2 or to 1/10 of the dissolved lipid extract of the effluent from the heart collected in 10 min periods corresponding in time to the bioassay tracing

rabbit heart observed release in the effluent of prostaglandin like substances However Minkes Douglas and Needleman (1973) reported that 5–8 min of ischemia only occasionally elicited release of prostaglandin like substances from the perfused rabbit heart Ischemia has earlier been shown to effect prostaglandin liberation from other tissues and species (McGiff *et al* 1970 b Jaffe *et al* 1972 Lonigro *et al* 1973) The present results support the hypothesis that ischemia in fact is a general stimulus for prostaglandin formation and release The current experiments further suggest that the proper stimulus for the prostaglandin release is the hypoxia induced during ischemia Changes in the perfusion conditions induced by ischemia like low perfusion pressure absence of glucose and acidosis did not elicit prostaglandin release in the current experiments and seem to be insignificant in this respect

If ischemia *via* the hypoxia induced is a general stimulus for the release of prostaglandins from tissues it seems possible on the basis of their activity as vasodilators that endogenously formed prostaglandins contribute to the development of reactive hyperemia Recent results in our laboratory have supported this hypothesis by showing that inhibition of prostaglandin synthesis in humans is followed by a decrease but not complete inhibition of the reactive muscle hyperemia in man (Kilbom and Wennmalm 1974)

The sympathetic neurotransmitter noradrenaline was found to be a potent stimulus for the liberation of prostaglandins in the present study thereby confirming in this organ earlier investigations in the spleen (Gilmore Vane and Wyllie 1968 Davies Horton and Withrington 1968) The inability of noradrenaline to release prostaglandins following alpha and beta adrenergic receptor blockade indicates that the drug when initiating prostaglandin

formation and release activates receptors functionally similar to the conventional alpha or beta adrenergic ones

Infusion of the parasympathetic neurotransmitter acetylcholine also elicited prostaglandin release from the heart When compared to the prostaglandin liberation produced by noradrenaline it appears that acetylcholine on weight basis has only about one third of the prostaglandin releasing capacity of noradrenaline The weaker capacity of acetylcholine to liberate prostaglandins is not necessarily explained in terms of a weaker ability of this drug compared to noradrenaline to activate receptors which initiate prostaglandin formation and release it might also be due to different concentrations in the myocard of the receptors for prostaglandin liberation activated by the two drugs

The releas of prostaglandins from the rabbit heart by noradrenaline has earlier been shown to constitute the basis for the prostaglandin mediated feed back inhibition of the release of the sympathetic neurotransmitter (Samuelsson and Wennmalm 1971) The current observation that acetylcholine is also capable to elicit prostaglandin release implies that a similar mechanism might be operating at the parasympathetic nerve endings as well It has earlier been shown that the process of release of acetylcholine from the parasympathetic nerve endings in the rabbit heart is inhibited by exogenously administered prostaglandins (Wennmalm and Hedqvist 1971) Whether the amount of prostaglandins physiologically released by parasympathetic nerve stimulation (Junstad and Wennmalm 1974) is sufficient to significantly restrict the liberation of neurotransmitter can not be judged at present and needs further investigations

In conclusion the experiments presented show that liberation of prostaglandins from the rabbit heart is elicited by a limited number of stimuli including hypoxia noradrenaline and acetylcholine It is shown that the mechanical performance of the heart is not correlated to the overflow of prostaglandins from the organ It is suggested that ischemia when stimulating prostaglandin formation and release from tissues operates *via* restriction of tissue oxygenation It is finally shown that acetylcholine possesses the ability to induce prostaglandin formation and release This ability might constitute the basis for an endogenous prostaglandin mediated feed back mechanism for restriction of the parasympathetic neurotransmitter

This study was supported by the Swedish Medical R search Council project 4341-01 a d by Magnus Bergvalls St ftelse The skilful technical assistance of miss Gun Lindgren is gratefully acknowledged

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Acta physiol scand 1975 93 25–33
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Role of the Symphato-Adrenal System in Hemorrhagic Hyperglycemia

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Received 13 May 1974

Abstract

JARHULT J *Role of the sympatho adrenal system in hemorrhagic hyperglycemia* Acta physiol scand 1975 *93* 25–33

Arterial and venous plasma glucose concentrat on was determined at intervals in cats subje ted to hemorrhagic hypotension at 50 mm Hg. The rapid rise of arterial plasma glucose after hemorrhage could be attributed to an increased release of glucose from the liver This hyperglycemia could not be eliminated by bilateral adrenalectomy or by sectioning of the hepatic sympathetic nerves although the response was somewhat depressed by the latter procedure On the other hand the hyperglycemia was virtually abol shed after adrenalectomy when combined with bilateral sectioning of the major and minor splanchnic nerves The level of plasma glucagon during hemorrhage increased in cats with an intact sympatho adrenal system but was unchanged in animals with combined splanchnic sympath-ctomy and adrenalectomy It is concluded that dur ng hemorrhage the sympatho-adrenal system influences the glucose output from the liver by three different reflex mechanisms (a) release of catecholamines from the adrenal glands (b) direct sympathetic nerve influence on the l ver and (c) release of glucagon from the pancreas

A marked increase of blood glucose after hemorrhage was first described by Claude Bernard in 1877 and his finding in dogs has later been repeatedly confirmed in many species including man during different hemorrhagic situations (*e g* Aub and Wu 1920 Brooks 1935 Halmagyi *et al* 1966 Carey Lowery and Cloutier 1970) The hyperglycemia during hemorrhage is mainly due to an increased release of glucose from the liver via glycogenolysis and it occurs already in early stages of bleeding (*e g* Shoemaker Walker and Turk 1961 Shoemaker *et al* 1973) It is commonly believed that adrenaline which increases severalfold in plasma during hemorrhage (*e g* Bedford 1917 Walker *et al* 1959 Carey Sapira and Curtin 1972) is the factor responsible for the hepatic glycogenolysis This conclusion is based on the finding that adrenalectomy can cause a decrease or abolition of the hemorrhagic hyperglycemia (*e g* Engel Winton and Long 1943 Halmagyi Irving and Gillett 1967 McCormick *et al* 1969) an effect present also after substitution of glucocorticosteroids (*e g* Hiebert *et al* 1973) and further on reports that ganglionic blockade can abolish the hyperglycemic response (Halmagyi *et al* 1966)

The hemorrhagic hyperglycemia has been shown to play an important role in the control of plasma volume during hypovolemia by causing an osmotic absorption of extra

vascular fluid to the blood stream (Järhult 1973) Some occasional observations in the latter study indicated however that a pronounced hemorrhagic hyperglycemia response could be present also after acute bilateral adrenalectomy suggesting that factors other than the adrenal hormones contributed to the blood sugar increase This opinion is supported by some other recent observations that stimulation of the sympathetic nerves to the liver or to the pancreas can cause a rapid and clearcut hepatic release of glucose (*e g* Edwards 197? Edwards and Silver 1972 *Bloom Edwards and Vaughan 1973*) *In view of these findings* it was considered worthwhile to analyse in greater detail the cause(s) of the hyperglycemia response in hemorrhage with regard to possible different sympatho adrenal influences on the reaction

Methods

33 cats (2 0–4 5 kg) of both sexes were kept on a normal mixed diet and fasted for 16 h before the experiment *After induction with ether the cats were anesthetized with chloralose (50 mg/kg) supplemented* initially by a small dose of pentobarbital sodium (5–15 mg) The animals breathed spontaneously throughout the experiment

Surgical preparation and material The abdomen was opened by a mid line incision to permit interference with the sympatho adrenal system and *inspection of catheter positions (see below) After heparinization* (750 IE/kg) two fine siliconized polyethylene catheters were inserted into the inferior caval vein via the two major saphenous veins to permit blood sampling One catheter was advanced under inspection until its tip was positioned just above the iliac bifurcation and the other one to a site just abo e the confluence of the inferior caval and the hepatic veins Th catheters were anchored by ligatures around the saphenous veins a *procedure found to keep the tips in correct position throughout the experiment In* seven of the experiments a fine catheter was inserted into the portal vein via cannulation of a distal branch of the superior mesenteric vein Hemorrhage was performed from the right carotid artery which was cannulated and connected via a catheter to a graded siliconized pressure bottle the pressure of which could be adjusted manually to desired levels From one T tube in this catheter the arterial blood pressure was *monitored continuously with a Statham P23 AC transducer on a Grass polygraph and from* another T tube art rial blood samples could be withdrawn

The animals were divided into 4 different groups In 14 cats the sympatho adrenal syst m was left intact (control animals) In 7 cats the adrenal glands were carefully removed bilaterally special caution being taken not to injure the adjacent main splanchnic nerves (adrenalectomized animals) In 6 cats the *hepatic sympathetic nerve plexus was dissected from around the hepatic artery and portal* vein in their passage through the hepato-duodenal ligament and the nerves were ligated and cut (animals with regional hepatic sympathectomy) In 6 expts the right and left major and minor splanchnic nerves were dissected free between the diaphragm and the adrenal gland ligated and cut In addition the adrenal glands were removed bilaterally (animals with splanchnic sympathectomy and adrenalectomy)

Experimental p otocol After the surgical procedures were finished the animals rested for about 30 min In this period blood samples were withdrawn for glucose osmolality and glucagon determinations (control samples) The animals were then bled rapidly to a mean arterial blood pressure of about 50 mm Hg a level which was maintained throughout the rest of the e periment with the aid of the pressure bottle Blood samples were taken 5 10 15 0 30 40 and 50 min after the start of the bleeding S multaneou ly blood sampling was made from three different sites From the carotid artery (arterial samples) from the distal caval catheter (iliac vein samples) and from the proximal caval catheter placed at the entrance of the hepatic veins (hepatic vein samples) In 7 of the cats sampling was made from the portal ein

Plasma osmolality was determined by thermistor cryoscopy (Osmometer 31 LAS Ad anced Instruments Inc) and plasma glucose concentration by the glucose-oxidase method the glucagon concentration (immuno-reactive glucagon) was estimated by a radio-immuno-assay technique described by *Nil son and* Uvnäs Wallensten (1974)

In Results section spread of data is gi en as S E. Significance tests were performed accordi g to the Student t test

Results

In an attempt to investigate whether the hemorrhagic hyperglycemia could be attributed to a hormone release from the adrenals to a sympathetic nervous influence on the liver and/or to a sympathetically induced release of glucagon from the pancreas the hyperglycemia response was analysed in animals with an intact sympatho-adrenal system and compared with that after adrenalectomy after regional hepatic sympathectomy and after splanchnic sympathectomy combined with adrenalectomy In addition the level of glucagon in the portal venous blood was determined For a rough evaluation of the role of the liver in the hyperglycemia response determinations of glucose were made in blood sampled simultaneously from the portal vein and from two sites in the caval vein *i e* at the hepatic venous confluence and at the iliac bifurcation As an extension of a previous study (Jarhult 1973) changes of plasma osmolality were also followed

It was recently shown that the hemorrhagic hyperglycemia in the intact cat develops quickly after the start of the bleeding and reaches a peak value in the arterial blood within about 20 min with a subsequent slow decline (Jarhult 1973) In the present investigation the duration of hemorrhagic hypotension (50 mmHg) was set so as to permit observation of this early hyperglycemia response In most cases the observation time was extended to 50 min after the commencement of the bleeding but a few animals died earlier which can be seen in Fig 2 in terms of the decrease in the number of observations (n) with time In the control period before bleeding mean arterial blood pressure averaged 121 ± 3 mm Hg for all animals There was no statistically significance difference between control blood pressure in the 4 experimental groups

Change of arterial glucose concentration

The arterial plasma glucose concentration in the control period before bleeding was not significantly different in the four experimental groups and averaged 210 ± 30 mg% Fig. 1 shows data during the hemorrhagic hypotension (50 mm Hg) in which the increase of arterial plasma glucose concentration above the control level is plotted against the time after the commencement of the bleeding It can be seen that the arterial plasma glucose concentration increased rapidly during hypotension in the control group (14 cats closed circles) to reach a peak value of 345 ± 68 mg% after 15 min after which it gradually decreased The adrenalectomized animals (7 cats open circles) responded quite similarly The data for the glucose rise were thus not significantly different from those in the control group In the 6 cats with regional hepatic sympathectomy (open squares) the initial (< 20 min) response was less pronounced than in the control and the adrenalectomized animals After 30 min of hemorrhagic hypotension however the increase of arterial plasma glucose concentration was as large as in the control animals In the 6 cats with splanchnic sympathectomy combined with adrenalectomy the response to bleeding was quite different as evidenced by the closed triangles in Fig 1 The average arterial glucose concentration was thus only slightly increased and three of these animals show no hemorrhagic hyperglycemia response at all

vascular fluid to the blood stream (Jarhult 1973) Some occasional observations in the latter study indicated however that a pronounced hemorrhagic hyperglycemia response could be present also after acute bilateral adrenalectomy suggesting that factors other than the adrenal hormones contributed to the blood sugar increase This opinion is supported by some other recent observations that stimulation of the sympathetic nerves to the liver or to the pancreas can cause a rapid and clearcut hepatic release of glucose (*e g* Edwards 1972, Edwards and Silver 1972 Bloom Edwards and Vaughan 1973) In view of these findings it was considered worthwhile to analyse in greater detail the cause(s) of the hyperglycemia response in hemorrhage with regard to possible different sympatho-adrenal influences on the reaction

Methods

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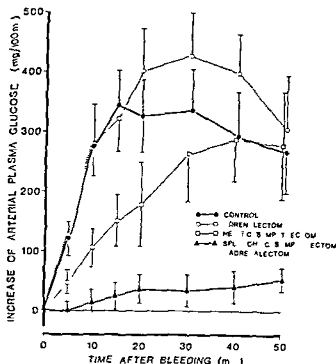


Fig. 1 Increases of arterial plasma glucose concentration above the control level during hemorrhagic hypotension at 50 mm Hg in control animals (n = 14) animals with adrenalectomy (n = 7) animals with regional hepatic sympathectomy (n = 6) and animals with splanchnic sympathectomy combined with adrenalectomy (n = 6) Mean values ± S E are given

Change of venous glucose concentration

a Iliac vein During normotension in the control period venous plasma glucose concentration in the iliac vein was quite similar in the four different groups and averaged 204 ± 18 mg° During hemorrhage the glucose concentration in the venous plasma rose gradually but not as rapidly as in the arterial blood The data for the different groups of animals are given in Fig 2 It may be noted that a clearcut positive arterio venous glucose difference existed during the first 30–40 min of hemorrhagic hypotension in agreement with previous observations (Jarhult 1973)

b Portal vein Plasma glucose concentration was determined in blood samples from the portal vein in 1–2 animals of each experimental group The data for the glucose increases in the portal venous blood were somewhat higher although not statistically different from those taken simultaneously from the iliac vein

c "Hepatic vein Blood samples taken from the site where the inferior caval and the hepatic veins confluence were considered to reflect semiquantitatively the glucose changes in the hepatic venous blood during hemorrhage The data obtained are summarized in Fig. 2 It can be seen that the glucose concentration in the hepatic venous blood clearly exceeded that in iliac venous blood and also that in arterial blood in all groups except the animals with splanchnic sympathectomy and adrenalectomy As a rule this glucose excess was most pronounced during the first 15 min of hemorrhage and then declined gradually The increase of glucose concentration in the "hepatic vein was clearcut already 2 min after the start of the hemorrhage in the control group — These data indicate that the hemorrhagic

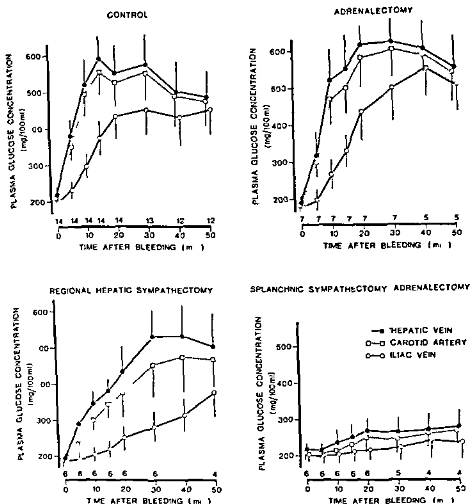
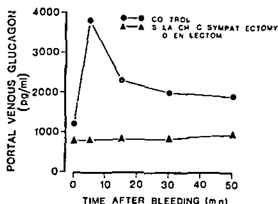


Fig 2 Simultaneously determined plasma glucose concentrations in the hepatic venous arterial and iliac venous blood for four groups of animals Control (n = 14) adrenalectomy (n = 7) regional hepatic sympathectomy (n = 6) and splanchnic sympathectomy + adrenalectomy (n = 6) Mean values ± S E given

hyperglycemia emanated from the liver and that the hepatic glucose release persisted after adrenalectomy and after regional hepatic sympathectomy although somewhat reduced in the latter group On the other hand the response was virtually abolished after splanchnic sympathectomy and adrenalectomy

Change of glucagon concentration

The level of immuno reactive glucagon in portal venous plasma was determined before and during hemorrhagic hypotension at 50 mm Hg in 2 cats with an intact sympatho-adrenal system and in 2 cats with splanchnic sympathectomy and adrenalectomy Fig 3 shows the mean data for glucagon concentration (panel A) and for arterial glucose



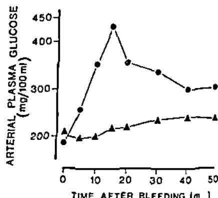


Fig 3 Mean values for portal venous glucagon concentration (panel A) and for arterial plasma glucose concentration (panel B) in 2 cats with an intact sympatho adrenal system and in 2 cats with splanchnic sympathectomy + adrenalectomy

concentration in simultaneously taken samples (panel B) It can be seen that hemorrhage caused a clearcut increase of the glucagon and glucose concentrations in the control animals with intact sympatho adrenal system and that these effects were virtually abolished by splanchnic sympathectomy and adrenalectomy These data indicate that glucagon is one of the factors which contributes to the hemorrhagic hyperglycemia and that this glucagon release seems to be mediated via the sympatho adrenal system The glucose concentration in hepatic venous blood was higher than in portal venous and iliac venous blood indicating that the glucose emanated from the liver also in these experiments

Change of plasma osmolality

It has previously been shown that hemorrhage causes a marked plasma hyperosmolality in cats with an intact sympatho-adrenal system which in turn is almost entirely due to the concomitant plasma hyperglycemia (Jarhult 1973) These results could be confirmed in the present study In addition it showed that the hemorrhagic hyperosmolality was unaffected by adrenalactomy somewhat reduced by hepatic sympathectomy and virtually abolished by splanchnic sympathectomy combined with adrenalectomy The hyperosmolar and the hyperglycemic responses changed in parallel in these 4 experimental groups

Discussion

The present study has shown that hemorrhage in the cat causes a rapid increment of arterial plasma glucose concentration due to an increased glucose release from the liver In contrast to some earlier observations (*e g* Engel Winton and Long 1943 Halmagyi *et al* 1966 Halmagyi Irving and Gillet 1967 McCormick *et al* 1969 Hiebert *et al* 1973) this study showed that the hyperglycemia was not abolished by removal of the adrenal glands

in fact the response seemed unaffected by bilateral adrenalectomy (Fig 1) However the hyperglycemia response tended to be somewhat depressed after regional sympathectomy of the liver at least in initial stages of hemorrhage and it was virtually eliminated after splanchnic sympathectomy combined with bilateral adrenalectomy (Fig 1) An increase of the plasma glucagon concentration noted in the intact animals during bleeding was no longer seen after splanchnic sympathectomy combined with adrenalectomy (Fig 3)

These results indicate that there are several mechanisms responsible for the hyper glycemia response in hemorrhage Thus reflex sympathetic effects directly on the liver and on the glucagon release from the pancreas seem to play important roles in the reaction but the experiments do not exclude that the adrenals perhaps mainly via adrenaline release also contribute to the response The magnitude of the hyperglycemia after selective interference with one or several of these links suggests that either of these mechanisms can relatively effectively substitute the others It should be pointed out however that the spread of data inherent in this type of experiment is quite large and that therefore an exact evaluation of the quantitative importance of each factor is difficult This problem is further complicated by the fact that the plasma glucose concentration in the control situation was high (≈200 mg%) which suggests that some of the glucose releasing mechanisms might have been activated more than in a true resting situation for instance by the stress caused by the surgery or anesthesia

The conclusion that catecholamine release is not the sole factor responsible for the hemorrhagic hyperglycemia is supported by a previous study by Carey Sapira and Curtin (1972) who found that it was necessary to infuse a dose of adrenaline 2 to 5 times greater than that released from the adrenals in hemorrhage to evoke a hyperglycemia in non bled animals corresponding to that seen in bled animals

The conclusion that there is a reflex sympathetic influence directly on the liver during hemorrhage resulting in a release of glucose is corroborated by earlier experiments on adrenalectomized animals in which the hepatic nerves were stimulated artificially (Edwards 1972 Edwards and Silver 1972) These authors found that a maximal stimulation (20 Hz) of the hepatic nerves in cats led to an increase of the arterial plasma glucose level by about 240 mg

The present finding indicating a sympathetically mediated reflex release of glucagon during hemorrhage is similarly supported by earlier stimulation experiments Thus excita tion of the sympathetic nerves to the pancreas in cats and calves led to a rapid mobilization of glucagon and to an increased plasma glucose concentration (Esterhuizen and Howell 1970 Bloom Edwards and Vaughan 1973) These two events were closely coordinated in time whereas in the present study the peak glucagon response clearly preceded the peak glucose response (Fig 3) The latter finding indicates that glucagon could not have been the sole cause of the hemorrhagic hyperglycemia in these experiments This opinion receives some further support by previous observations that physiological stimulation of the splanchnic nerves in adrenalectomized animals with severed hepatic nerves increased the plasma glucose level by only 40 mg% (Edwards and Silver 1972) This value in turn may be compared with the 290 mg% increase of glucose noted during bleeding in animals with sectioned hepatic nerves (Fig 1) These data suggest that besides the glucagon mechanism

the adrenal hormones could have contributed to the hemorrhagic hyperglycemia There seems to be little doubt however that the reflex glucagon mechanism is quite an important factor contributing to the hyperglycemia in early stages of bleeding Glucagon can apparently be released also in late phases of hemorrhage as evidenced by Halmagyi *et al* (1969) They found an increased glucagon concentration 3 hours after hemorrhage but this increase was considered to be secondary to the hypoglycemia often found in late stages of shock

Splanchnic sympathectomy combined with adrenalectomy markedly depressed the hemorrhagic hyperglycemia but the response was not completely abolished (Fig 1 and 2) The increase of arterial plasma glucose concentration averaged 40 mg% after 20 min of hemorrhage This effect may be due to an incomplete splanchnic denervation or to activation of other glucose releasing mechanisms for instance via growth hormone (e g Meyer and Knobil 1967 Carey Cloutier and Lowery 1971 Skillman *et al* 1971) or via interference with the insulin secretion (for ref see below)

The present multifactorial hypothesis for the glucose release during hemorrhage contrasts as mentioned above to some earlier studies which have attributed the hyperglycemia solely to an increased secretion of adrenaline It is important to point out in this context that changes in the carbohydrate metabolism during hemorrhage are easily influenced for instance by the rate of bleeding (Carey and Wallack 1970 Hall and Hodge 1971 Carey Sapira and Curtin 1972) the volume of the shed blood (e g Walker *et al* 1959) and differences in the experimental approach etc (e g Chien 1967) Another problem in interpreting and comparing the results from different investigations is the considerable variation of the hemorrhagic hyperglycemic response even in the intact animal a phenomenon which at least partly may be explained by interindividual differences in the amount of stored glycogen in the liver (Engel Winton and Long 1943) Species differences may also very well exist As an example of this the insulin response during hemorrhage may be mentioned Insulin has been shown to be unaffected or to decrease in primates including man (e g Moss *et al* 1970 Carey Lowery and Cloutier 1970 Cerchio *et al* 1971) whereas it increases in the dog (e g McCormick *et al* 1969 Vigas *et al* 1972)

The hyperglycemic response undoubtly forms an important compensatory mechanism in hemorrhagic hypovolemia For instance it must help to improve the nutritional situation for the tissues during blood flow reduction and in addition it serves as an important factor in the plasma volume control The later mechanism is mediated via the resulting arterial hyperosmolality which causes considerable osmotic absorption of fluid from the extravascular space to the blood stream (Järhult 1973) It seems adequate that such a vital compensatory mechanism is controlled by several different links

The author want to thank Miss Ann Ch stin Isaksson M ss Margaretha Rahm and Mrs Tora Zad v Johansson for in aluable technical ass stance during the work Dr Goran Nilsson has kindly performed the glucagon det rminations The study was supported by grants from the Swedish Medical Research Council (B75 14X 10-09A) and from the Faculty of M dicine University of Lund (5148 31 ? 3)

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Acta physiol scand 1975 93 34-51
From the Department of Physiology University of Göteborg Sweden

Circulatory Responses to Stimulation of the Carotid Body Chemoreceptors in the Cat

By

ROD LITTLE[1] and BENGT ÖBERG

Received 13 May 1974

Abstract

LITTLE R and B ÖBERG *Circulatory responses to stimulation of the carotid body chemoreceptors in the cat* Acta physiol scand 1975 *93* 34-51

Cardiovascular responses to carotid body chemoreceptor stimulation were followed in the curarized vagotomized artificially ventilated cat Stimulation of the chemoreceptors by perfusion of the carotid sinus regions with venous blood induced a reflex vasoconstriction in skeletal muscle kidney intestine and skin and in most cases an increased heart rate A comparison of the chemor ceptor reflex responses with those obtained by direct electrical stimulation of the regional vasomotor fibres indicated that in chemoreceptor reflexes the vasomotor fibre activity increased to the same extent in skeletal muscl and intestinal resistance vessels and probably in the nutritional skin vessels but to a smaller extent in the renal vessels and the skeletal muscle capacitance vessels The renal vessels and the muscle capacitance vessels seemed however to respond more during chemoreceptor stimulation than when the baroreceptors were unloaded indicating that an excitation of the bulbar vasomotor centre is more effective than the withdrawal of an inhibitory restraint in activating the vasomotor fibres to these vascular sections

The reflex circulatory adjustments following chemoreceptor stimulation have been widely studied (*e g* Bernthal 1938 Bernthal and Schwind 1945 Daly and Scott 1962 1963 Daly and Ungar 1966 Downing Remensnyder and Mitchell 1962 Calvelo *et al* 1970 Pelletier 1972) The reflex responses elicited experimentally appear to be influenced both quantitatively and qualitatively by the buffer nerves by artificial or spontaneous ventilation and the arterial P_{CO} It seems to be generally accepted however that when the chemoreceptors are allowed to exert their action undisturbed by simultaneous influences from lung receptors baroreceptors etc they induce an increased sympathetic vasoconstrictor fibre discharge to the vascular beds and an increased vagal outflow combined with a reduced cardio-accelerator fibre activity Recently a non-cholinergic vasodilatation in the skin in response to chemoreceptor stimulation has been reported (Calvelo *et al* 1970)

Few attempts however have been made to analyze quantitatively the involvement of

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the various vascular beds in chemoreceptor reflexes Bernthal and Schwind (1945) noted that the muscle vessel responded more vigorously than the intestinal vessels following a mild stimulation of the chemoreceptors Whether this difference was due to a non uniform activation of the vasomotor fibres to the two vascular beds or to differences in the effector response to a given increase of the vasomotor fibre activity was not resolved in their experiments

In the present study the chemoreceptor influences on the resistance vessels in various parallel-coupled vascular beds such as skeletal muscle skin kidney and intestine have been studied as well as the reflex responses of the capacitance vessels in the skeletal muscles The size of the reflex responses was compared with the effects induced by an unloading of the arterial baroreceptors and in many experiments with the responses to direct electrical stimulation of the regional vasoconstrictor fibres

Methods

Experiments were performed on 32 cats anaesthetized with chloralose (30–50 mg kg b wt i v) after ether induction A tracheal cannula was inserted and the carotid arteries and the vagus nerves were freed bilaterally in the neck All main side branches of the common carotid arteries and the external carotid arteries in the vicinity of the sinuses were ligated and divided. To avoid damage to the carotid body circulation and to the innervation of the carotid sinuses no attempt was made to ligate all the minor side branches in this region

Recording of circulatory variables

Arterial blood pressure was measured from one brachial artery with a Statham P23AC transducer on a Grass Model 5 Polygraph recorder *Heart rate* was monitored by a tachograph triggered by the rapid *systolic upstroke of the arterial blood pressure In most experiments the arterial blood pressure was kept* constant by connecting the arterial side of the circulation (via the femoral the superior mesenteric or the central end of one carotid artery) to a blood filled syringe serving as a pressure reservoir

Calf muscle blood flow was measured in all 32 expts by cannulating the deep femoral vein (or popliteal vein) and diverting the venous outflow through an optical drop chamber operating an ordinate writer on the polygraph The blood was usually returned through the opposite femoral vein *Renal blood flow* was measured in 14 cats The abdomen was opened the intestine removed and the left renal vein freed and cannulated After passing the drop chamber the venous outflow from the kidney was returned to the animal via the superior mesenteric vein *Intestinal blood flow* was recorded in 4 animals After opening the abdomen a segment of jejunum weighing 30–40 g, was isolated and the rest of the intestine removed The superior mesenteric vein was cannulated and after passing the drop chamber the blood was returned to the animal usually via the left renal vein *Skin blood flow* was measured in 5 animals by *recording the* outflow from the superficial saphenous vein which was cannulated at the level of the ankle joint. In 3 of the cats skin vascular responses were followed before and after occlusion of the circulation to the pads by forceps *Capacitance vessel responses* in the calf skeletal muscles were followed in 9 animals For this purpose the calf was prepared as described by Kjellmer (1964) *All tissues except for the femur the* popliteal artery and vein and the sciatic nerve were severed at the level of the knee joint The femoral marrow cavity was carefully plugged to obstruct possible venous drainage from the muscle The preparation was placed in a plethysmograph, filled with water at 37 C and connected to a volume recording device Volume changes were recorded on the polygraph recorder The sensitivity was adjusted so that a volume change of 0 1 ml gave a pen deflection of around 10 mm The popliteal vein was cannulated and the venous outflow recorded by a drop chamber-ordinate writer unit. The blood was returned to the animal via the opposite femoral vein

The responses of the resistance vessels in the various circuits are given as changes in flow resistance (PRU) defined as $\frac{\text{pressure (mm Hg)}}{\text{flow (ml/min)}}$ The calf muscle capacitance vessel responses were determined from the rapid phasic changes in tissue volume (see Mellander 1960)

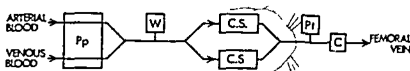


Fig. 1 Diagrammatic representation of carotid sinus perfusion system P_p, Harvard peristaltic pump (Model 500–1?00) W Windkessel reservoir C S carotid sinuses Pt pressure transducer C, adjustable screw clamp

Stimulation of the chemoreceptors

The prepared carotid sinus regions including the carotid bodies were perfused with arterial venous or mixed arterial/venous blood using the perfusion system shown diagrammatically in Fig 1 For arterial perfusion one artery usually the superior mesenteric or one carotid artery was cannulated and the blood passed through one channel of the pump For venous perfusion the right ventricle was catheterised via the right jugular vein and the blood passed through the second channel of the pump Intra sinusal pressure and hence baroreceptor activity could be controlled by a screw clamp placed distal to the site of pressure recording (Fig. 1)

In the present experiments no attempt was made to graduate the chemoreceptor stimulations The extent of activation of the receptors was determined by the differences in pH P_{CO} and P_{O} of the arterial and venous blood perfusing the sinus regions and was thus the result of a combined reduction of pH and P_{O} and increase of P_{CO_2}, as shown in Table I These values were obtained from repeated measurements in 16 animals by means of a Radiometer Type 27 apparatus The low arterial pH values often found in these animals were subsequently corrected by adjusting the tidal volume in the animals ventilated with room air and by intravenous administration of bicarbonate in animals given 3 CO_2—It seems likely that with the reduced peripheral blood flow during chemoreceptor stimulation there will be a progressive fall of pO_2 and pH and an increase of pCO_2 in the mixed venous blood returning to the right ventricle As blood was taken from the right ventricle for venous perfusion of the sinus regions a progressively intensified chemoreceptor stimulation and effector response was often seen during the period of venous ... on The values for pH etc given in Table I come from venous samples taken in the later stages of venous perfusion

Stimulation of the regional vasomotor fibres

The regional vasoconstrictor fibres to the respective vascular beds i.e. the lumbar sympathetic chains (calf muscle and skin preparations) and the postganglionic fibres running along the superior mesenteric artery were carefully freed for a short distance and placed on electrodes The nerves which were left with intact central connections were bathed with liquid paraffin Supramaximal stimuli were delivered at rates varying between 1–6 Hz.

With the mentioned technique of vasomotor fibre stimulation impulses may also pass in afferent direction to the vasomotor centre so that, besides the direct electrical stimulation there was also a *reflex* augmentation of vasoconstrictor fibre activity However such undesirable reflex effects were evidently of no importance in the present study since significant reflex blood pressure and muscle vessel responses to *e.g.* mesenteric nerve stimulation were observed first with stimulation frequences which were decidedly higher than those presently used to induce regional vascular responses *i.e.* well above 6 Hz

Experimental procedures At the start of the experiment the animals were *curarized* (Flaxedil mg/kg b wt) and placed on artificial respiration. The animal was either ventilated with room air or more often with a gas mixture containing 3 CO in 97 O_2 Both vagal nerves including the aortic nerves were cut in the neck The carotid sinus regions were perfused initially with arterial blood and the sinus pressure set at around 100 mm Hg. After a control period venous perfusion of the sinus regions was started care being taken to keep the intrasinusal pressure unchanged Due to the dead space of the tube system there was a delay before the venous blood reached the sinus regions The arrival of the venous blood at the regions could however be easily observed by the colour of the blood in the transparent plastic tubing

Following a – 5 min period of venous perfusion during which time steady state vascular responses were usually obtained arterial perfusion was restarted With a return to control conditions the intrasinusal

TABLE I

	Ventilation with room air		Ventilation with 97 O + 3 CO	
	Arterial blood (n = 14)	Venous blood (n = 16)	Arterial blood (n = 14)	Venous blood (n = 17)
pH	7 34 (7 7–7 41)	7.20 (7 05–7 36)	7 6 (7 15–7.38)	7 19 (7 11–7.30)
PCO_2, mm Hg	33 (24–40)	41 6 (29–67)	41 (3–48)	54 (38–59)
PO_2 mm Hg	98 5 (71–1 1)	38 (32–49)	337 (60–5 0)	53 (43–66)

Compos tion of arterial and venous bloods, perfusing the sinus regions in 8 animals ventilated with room air and in 8 animals ventilated with a gas mixture of 97 O and 3% CO_2. Mean values and range of variation gi en n = number of samples

pressure was lowered and the steady state reflex vascular responses, resulting from the diminished baroreceptor activity observed The responses to regional vasomotor fibre stimulation were subsequently analyzed

Heparin 5 mg kg, was used as anticoagulant.

Results

Stimulation of the carotid body chemoreceptors by venous perfusion of the carotid sinus regions produced a reflex increase of blood pressure (in experiments where the blood pressure was allowed to change) an increase in heart rate and a generalized vasoconstriction in the vagotomized curarized and artificially ventilated cat where the buffering influences from the arterial baroreceptors were eliminated Although the magnitude of the responses differed substantially between the different animals they were always present whenever the bulbar vasomotor centre was responsive as demonstrated by the reflex circulatory responses to a reduced baroreceptor activity

Skeletal muscle resistance vessel responses

Chemoreceptor stimulation produced a constriction of the skeletal muscle resistance vessels The increase in flow resistance averaged 140 % above control in 145 tests in the 32 animals (range 25% to 770) measured as the maximal response obtained during the period of chemoreceptor stimulation The time course of the response differed somewhat in the individual tests Usually there was an early peak constrictor response which subsided within the first minute to a less intense steady-state constriction In other cases the constriction was well maintained or even showed a slow progressive increase throughout the period of stimulation such that no clear steady state situation was attained The constriction rapidly disappeared when arterial perfusion of the carotid sinus regions was restarted in many cases a period of reactive hyperaemia was seen particularly when the flow had been severely restricted during the preceding period of chemoreceptor stimulation

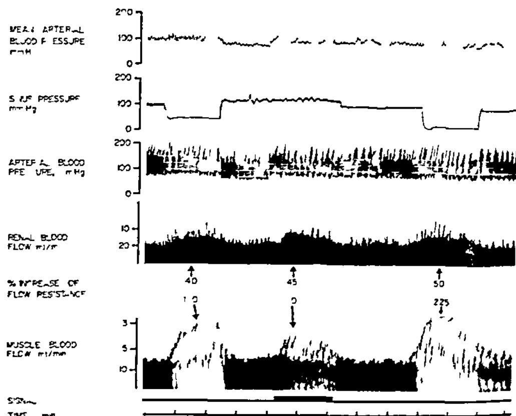
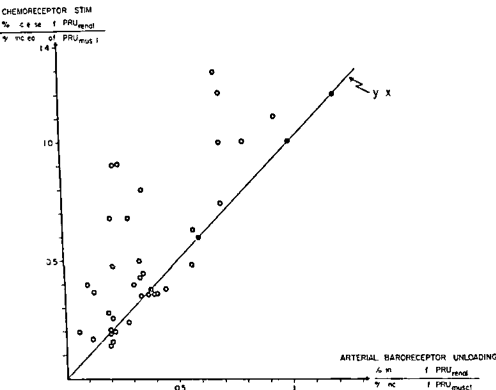


Fig. 2. Effects of baroreceptor unloading (lowering of the sinus pressure) and chemoreceptor stimulation (signal) on the renal and the skeletal muscle vascular beds. Blood pressure maintained constant throughout

Renal vascular responses

Renal vessel responses to chemoreceptor stimulation were studied in 58 tests in 14 animals. In all cases there was an increase in renal flow resistance averaging 55% above control (range 25–275 %). The renal vessel response usually developed more slowly than that in skeletal muscle, reaching a plateau value after 20–40 s of venous perfusion of the carotid sinus regions. Switching to arterial perfusion led to a rapid vasodilatation in the kidneys; reactive hyperaemia was rarely seen.

The renal vessel adjustments to chemoreceptor stimulation were compared in all animals with the responses obtained when the baroreceptor inhibitory restraint on the bulbar vasomotor centre was reduced or removed by lowering the sinus pressure. In such comparisons the muscle vessel responses obtained at the same time were used as a reference to describe the extent of vasomotor centre excitation in the two reflex mechanisms. A record illustrating one experiment of this type is shown in Fig. 2. It can be seen that although both chemoreceptor stimulation and lowering of the sinus pressure induce constriction of the two vascular beds, the relationship between the size of the responses in the two beds differs. While in this particular animal the renal responses were the same throughout the experiment (40–50% increase in flow resistance), the muscle vessel responses were signi-



F g 3 Comparisons of the renal and skeletal muscle vessel responses to chemoreceptor stimulation and baroreceptor unloading tests performed immediately after each other in the course of he e periment (paired tests) To normalize the data from all such comparisons the reflex effects are expressed as the ratio between renal and muscle vessel responses Each paired comparison is represented by one symbol It is seen that the data are displaced to the left and above the identity line indicating that for a gi en reflex muscle vessel re-ponse the renal vessel responses ar more pronounced when the chemoreceptors are stimulated than when the baroreceptors are unloaded If the angle with the x acxis by the l ne from the origin to each individual point is calculated the mean angle is found to be 50 94 $\pm$2 1 wh ch is statistically d fferent ($p<0\,01$) from the angle of the identity line (45)

ficantly smaller when the chemoreceptors were stimulated If the ratios of the renal to the muscle resistance vessel responses are calculated during chemoreceptor stimulation and baroreceptor unloading values from 1 4 to 1 4 5 and 1 1 1 respectively are obtained

The results of similar comparisons in 14 expts are shown graphically in Fig 3 The ratio between the renal and muscle vessel responses obtained from paired chemoreceptor and baroreceptor tests are here related to each other It is seen that in the majority of the cases the symbols fall above the line of identity ($y=x$) The data shown in Fig 3 were statistically treated in the following way The angle made with the x axis by the line from the origin to each individual point was measured and the mean angle compared in a t test with the angle for the identity line (45) The mean angle for the test data was found to be 50 9 $\pm$2 1 S E which was statistically different ($p<0\,01$) from 45 Thus for a given muscle vessel response the renal vessel response is significantly greater when the vasomotor centre is excited by chemoreceptor stimulation than by a reduced baroreceptor restraint

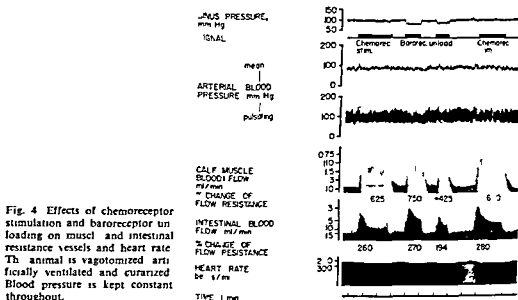


Fig. 4 Effects of chemoreceptor stimulation and baroreceptor un loading on muscl and intestinal resistance vessels and heart rate Th animal is vagotomized arti ficially ventilated and curarized Blood pressure is kept constant throughout.

It should be noted that in Fig 3 and subsequent figures the mean angle is calculated to allow for a statistical comparison of the position of the different groups of data in the diagram and does not imply that there is necessarily a linear correlation which could be described by the line passing through the origin and making the stated angle with the x axis

Intestinal vascular responses

The intestinal resistance vessel responses to chemoreceptor stimulation were studied in 19 tests in 4 animals and compared with the reflex effects in the calf muscle resistance vessels There was a reflex increase in intestinal vascular resistance averaging 177% above control and varying between 20% to 440 % in individual tests The responses were characterized by an initial pronounced constriction followed by a gradual diminution of the response despite continued receptor stimulation a so-called autoregulatory escape (Folkow *et al* 1964) The responses were calculated from the initial peak response The reflex effects of chemoreceptor stimulation were regularly compared with the reflex responses to a lowered baroreceptor activity In 2 animals comparisons were also made with the responses obtained with electrical stimulation of the regional vasomotor fibres

The record from a representative experiment is shown in Fig 4 It is seen that there was a vasoconstriction in both vascular beds in both reflex mechanisms and to judge from the relationship between intestinal and muscle vessel responses the intestinal vascular bed seems to be engaged to approximately the same extent in the two reflexes Thus the ratios between intestinal and muscle responses expressed as per cent increase of flow resistance were 1 2 3 when the chemoreceptors were stimulated and 1 2.5 when the baroreceptor activity was lowered The results of such comparisons of intestinal and muscle vessel responses to chemoreceptor and baroreceptor tests performed close in time in one and

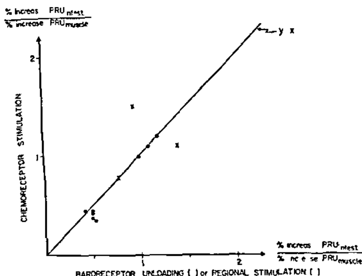


Fig. 5 The relation between the rat os of intestinal and muscle vessel responses, obtained with paired chemoreceptor and baroreceptor tests (filled circles) and paired chemoreceptor and regional aso constrictor fibre stimulation tests (crosses) respectively The mean angle with the x axis by the lines through the origin and the individual chemoreceptor/baroreceptor data was 44 63 ± 1 78 and the mean angle by the lines through the origin and the chemoreceptor/regional stimulation data was 45 -- ∓ 4 2 These angles are not statistically different from each other (p = 0 89) and neither of the angles is statisti cally different from 45 (p = 0 83 and 0 96 respectively) Therefore for a given muscle vessel response the intestinal vessel responses are of the same order of magn tude irrespecti e of whether the vasomotor fibres a e directly stimulated or reflexly acti ated by chemoreceptor stimulation or baroreceptor unloading

the same animal in all experiments in this series are summarized in Fig 5 In this figure which is constructed in the same way as Fig 3 the ratios between the intestinal and muscle flow resistance responses obtained with paired chemoreceptor and baroreceptor tests have been plotted against each other The ratio between intestinal and muscle vessel response following stimulation of the regional vasoconstrictor fibres with identical frequencies (see Methods) on one hand and following a paired chemoreceptor stimulation on the other has also been correlated (Fig 5) Only a limited number of tests of the latter kind were entirely successful The figure shows that the ratios between intestinal and muscle vessel responses are closely similar irrespective of whether the chemoreceptors are stimulated the baroreceptors unloaded or the regional vasomotor fibres activated *i e* the symbols are fairly equally dispersed on both sides of the identity line A statistical analysis of the data similar to that described above for the renal vessel responses revealed that the mean angle for the lines running from the origin to the individual points relating baroreceptor and chemoreceptor tests was 44 63 ± 1 78 This is not statistically different from the angle of the identity line (45) (p = 0 84) implying that the ratio between intestinal and muscle vessel responses is the same irrespective of whether the baroreceptors are unloaded or the chemoreceptors are stimulated It therefore seems as if the intestinal vascular bed is engaged to the same extent when the chemoreceptors are stimulated and the baroreceptors unloaded again using the muscle vessel responses as a measure of the vasomotor centre excitation in

the two cases A similar analysis of the data relating the effects of regional vasomotor fibre stimulation to the effects of chemoreceptor stimulation shows that the mean angle for the lines passing through the origin and the individual data is 45.22 ±4.27 This value is not statistically different from the angle of the identity line (45) ($p = 0.96$) nor from the above figure of 44.63 ±1.78 obtained when the baroreceptor and chemoreceptor responses were correlated ($p = 0.89$) Therefore the vasomotor fibres to the intestinal vessels and to the muscle vessels seem to be activated to the same extent in chemoreceptor reflexes just as previously found in baroreceptor reflexes (Kendrick *et al* 1972)

Skin vascular responses

The effects of chemoreceptor stimulation on paw circulation were studied and compared with the effects of baroreceptor unloading in 5 cats (24 tests) The regional vasomotor fibres were stimulated in 3 animals The response to chemoreceptor stimulation was invariably vasoconstriction flow resistance increasing on average by 119 % (range 20%–500%) Vasodilatation was never observed

Fig 6 shows records from a representative experiment in this series In the left panel the paw and muscle vessel responses to chemoreceptor stimulation baroreceptor unloading and regional vasomotor fibre stimulation are shown The paw and muscle vessel responses are seen to be approximately the same when the vasoconstrictor fibres are reflexly activated via the chemo and baroreceptors while a direct stimulation of the vasomotor fibres induces a much more pronounced constriction in the paw

In the right panel the pad circulation is excluded from the paw circulation by placing forceps around the bases of the pads This manœuvre caused an instantaneous marked reduction of paw flood flow (not seen in the figure) which however subsequently increased again so that after 2–3 min the flow resistance was only approximately 25% higher than before the obstruction (see Fig 6) The average increase of flow resistance in the steady state situation after obstruction of pad circulation was in the present 5 animals *31* ±*14* (range 16%–60%) After clamping of the pads the marked discrepancy between the effects of reflex and direct activation of the vasomotor fibres which was so clearly demonstrated in the intact preparation (left panel) is not seen Now the muscle vessels respond at least as much as the paw vessels to the regional vasomotor fibre stimulation

Data from all experiments of the type illustrated in Fig 6 (left panel) are shown in Fig 7 where the ratios between paw and muscle vessel responses observed in paired baroreceptor and chemoreceptor tests and paired direct stimulation and chemoreceptor stimulation tests are plotted against each other The symbols relating the baroreceptor and the chemoreceptor responses (dots) are seen to be evenly dispersed around the identity line while the data *relating the effects of direct stimulation to the responses to chemoreceptor stimulation* (crosses) are placed clearly below and to the right of the identity line The mean angle for the baroreceptor-chemoreceptor data is 47.8 ±1.9 which is not statistically different from 45 ($p = 0.16$) while the corresponding figure for the direct stimulation-chemoreceptor activation data is 18.6 ±1.9 which is statistically different from 45 (p 0.0001)

Thus if the muscle vessel responses are taken as a measure of the extent of activation of the vasomotor centre it seems as if the vasomotor fibres to the paw vessels are activated

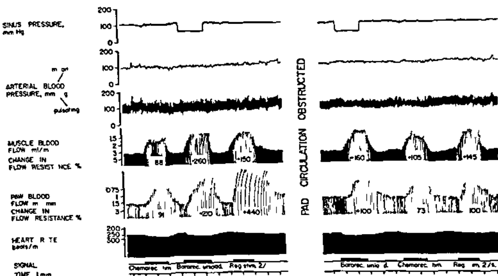


Fig 6 Left panel muscle and skin (paw) resistance vessel and heart rate responses to baroreceptor unloading chemoreceptor stimulation and direct vasomotor nerve stimulation Right panel the same procedures repeated after obstruction of the circulation through the pads Note the considerably smaller skin vessel responses to regional stimulation in this latter situation

to the same extent when the chemoreceptors are stimulated and the baroreceptors are unloaded On the other hand the reflex paw vessel responses are significantly smaller than those observed with direct stimulation of the regional vasomotor fibres This may be because the arterio venous anastomoses in the pads (which are involved in thermoregulatory mechanisms) are not reflexly influenced from baroreceptors and chemoreceptors but of course constrict when the regional vasomotor fibres are stimulated This interpretation is supported by the fact that when the pad circulation was excluded from the paw preparation the quotient between the paw vessel and muscle vessel responses became essentially the same when the vasomotor fibres were either directly stimulated or reflexly activated from the chemoreceptors This latter finding again suggests that the vasomotor fibres to the nutritional skin vessels and to the muscle vessels are activated to the same extent in chemoreceptor reflexes as has previously been shown to be the case in baroreceptor reflexes (Kendrick *et al* 1972)

Capacitance vessel responses in skeletal muscle

The responses of the calf muscle capacitance vessels to chemoreceptor stimulation were studied in 6 animals and routinely compared with the effects of baroreceptor unloading and electrical stimulation of the regional vasomotor fibres A record from one experiment in this series is shown in Fig 8 Chemoreceptor stimulation induced not only a constriction of the muscle resistance vessels but also a capacitance vessel constriction as reflected in the initial rapid decrease of tissue volume Qualitatively similar effects were produced by regional vasomotor fibre stimulation and baroreceptor unloading although quantitative differences

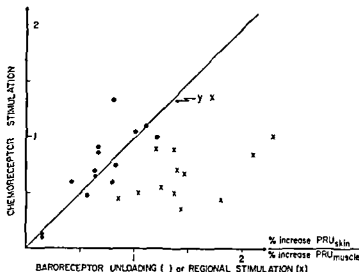


Fig 7 The relation between the ratios of muscle and skin vessel responses obtained with paired chemoreceptor and baroreceptor tests (filled circles) and paired chemoreceptor/regional vasomotor fibre stimulation tests (crosses) respectively The mean angle with the x axis by the lines combining the baroreceptor/chemoreceptor points with the origin was 47 8 ± 1 94 which is not different from 45 (p = 0 16) The mean angle for the lines combining the origin with the chemoreceptor/regional stimulation data was 18 6 ± 1 9 which is statistically different from 45 (p < 0 0001) and from 47 8 ± 1 94 (p < 0 0001)

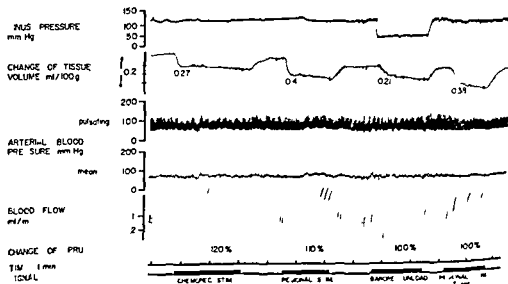


Fig. 8 Effects of chemoreceptor stimulation baroreceptor unloading and regional vasomotor fibre stimulation on muscle resistance and capacitance vessels The capacitance vessel responses are deduced from the initial fast reduction in tissue volume

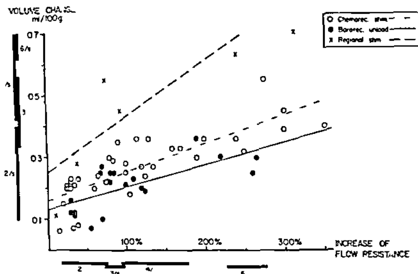


Fig 9 The relation between the muscle capacitance and resistance vessel responses to chemoreceptor stimulation (open circles) baroreceptor unloading (closed circles) and regional vasomotor nerve stimulation (crosses) The rates of stimulation (regional vasomotor fibre stimulation) are also gi en The regression lines for the chemoreceptor (y=0 0009x+0 15 r=0 8) and baroreceptor (y=0 0007x+0 13 r=0 69) data are statistically d fferent (p<0 005) (*cf* Brownlee 1965 p 349) The data obtained with direct stimula tion of the regional vasomotor fibres (y=0 0018x+0 ,5 r=0 81) are clearly displaced abo e and to the left of the two groups of reflex data (p<0 0001)

were seen The venous response was more pronounced when the regional vasomotor fibres were directly stimulated than when their activity was reflexly increased It was also seen that chemoreceptor stimulation leads to a slightly greater capacitance vessel response than an unloading of the baroreceptors despite similar resistance vessel responses in the two situations In Fig 9 data from all experiments in this series have been sum marised The venous responses expressed as tissue volume changes obtained with chemoreceptor stimulation baroreceptor unloading and regional vasomotor fibre stimulation are plotted against the resistance vessel responses obtained at the same time The stimulation rates used when the vasomotor fibres were stimulated directly are also shown The scatter of the data illustrates the variations in vessel responsiveness in individual experiments It is evident however that the venous responses for a given increase of flow resistance are more pronounced following direct stimulation than when the fibres are reflexly activated When regression lines are drawn it is found that the chemoreceptor stimulation data are displaced above those obtained by baroreceptor unloading suggesting that the venous responses to chemoreceptor stimulation are slightly more pronounced than when the receptors are unloaded

Heart rate responses

The reflex changes in heart rate in response to chemoreceptor stimulation were followed in 19 vagotomized curarized and artificially ventilated cats In 78 out of 99 chemoreceptor tests there was a moderate increase in heart rate (*cf* Fig 3 and 5) amounting to 3–5 ₒ

above control In two cats the increase in heart rate was more pronounced amounting to 20% and 25% above control respectively In 21 chemoreceptor tests there was no noticeable alteration of the heart rate Slowing of the heart in response to chemoreceptor stimulation was never observed A clearcut reflex slowing of the heart was however regularly produced when the intrasinusal pressure was elevated indicating a tonic cardioaccelerator fibre activity which could be reflexly inhibited Thus in the present experiments there was no evidence of a reflex inhibition of the accelerator fibre activity during chemoreceptor stimulation but rather of a reflex augmentation of the symptathetic influence on the heart

Blood pressure responses

The arterial blood pressure which was maintained constant throughout most of the experiments (see Methods) was allowed to change in response to chemoreceptor stimulation in 23 expts in 5 cats There was always a reflex rise in mean arterial blood pressure from an average control value of 93 mm Hg (range 75–123 mm Hg) to an average maximal value of 153 mm Hg (range 95–200 mm Hg)

Discussion

In the present study the circulatory adjustments to stimulation of carotid body chemoreceptors have been analyzed in the curarized artificially ventilated cat where the vagal nerves (including the aortic depressor nerves) were cut and the pressure in the carotid sinuses maintained constant Consequently chemoreceptor function was not disturbed by concomitant shifts of baroreceptor or lung inflation receptor activity or by changes in the chemical composition of the blood The results show that chemoreceptor stimulation induces a reflex constriction of all vascular sections studied *i e* the resistance vessels in skeletal muscle kidney intestine and skin and of the venous capacitance vessels in skeletal muscle There was also a slight increase in cardioaccelerator fibre activity in the vagotomized animals

In this study an attempt has been made to compare systematically the reflex circulatory effects ensuing when the medullary vasomotor centre was exposed to *excitatory* influences from the chemoreceptors on one hand and to a reduced *inhibitory* restraint from the arterial baroreceptors on the other When comparing the effects of chemoreceptor stimulation and baroreceptor unloading the skeletal muscle resistance vessels were used as a reference to describe the extent of overall excitation of the vasomotor centre in the two cases The results of such comparisons indicate that the renal vessel responses are somewhat more pronounced when the chemoreceptors are stimulated than when the arterial baroreceptor activity is lowered Also the muscle *capacitance* vessel responses are more pronounced in chemoreceptor than in baroreceptor reflexes These findings imply that a withdrawal of tonic baroreceptor inhibition on the medullary vasomotor centre is somewhat less effective in increasing the vasoconstrictor fibre activity to the renal vessels and muscle capacitance vessels than the addition of excitatory influences from the chemoreceptors

Similar comparisons between baroreceptor and chemoreceptor reflex influences on intestinal and skin vessels showed that the two reflex mechanisms were equally potent in

producing vasoconstrictor responses i e a given reflex muscle vessel response was accompanied by intestinal and skin vessel responses which were *of equal size irrespective* of whether the baroreceptors were unloaded or the chemoreceptors stimulated In these cases elimination of an inhibitory restraint on the vasomotor centre was evidently as effective as the introduction of excitatory influences

Another object of the present study was to estimate the involvement of various cardiovascular effectors in chemoreceptor reflexes by evaluating in approximate terms the reflex increase of vasomotor fibre discharge in various vascular beds when the chemoreceptors were stimulated This was done by comparing the reflex responses with the effects of direct electrical stimulation of the regional vasomotor fibres with known frequencies The skeletal muscle resistance vessel responses were again used as a reference to describe the extent to which the bulbar vasomotor centre was excited from the chemoreceptors The results from these studies showed that chemoreceptor stimulation increased the vasoconstrictor fibre discharge to the same extent in both the intestinal and muscle resistance vessels This is also true in baroreceptor reflexes (Kendrick *et al* 1972) *In contrast the muscle capacitance vessels and the paw vessels were found to be less strongly influenced* than the muscle resistance vessels when the chemoreceptors were activated Similar modest reflex responses of paw vessels and muscle capacitance vessels are also seen when the baroreceptor activity is reduced (Kendrick *et al* 1972, Hadjiminas and Oberg 1968) The apparent limited engagement of the paw circulation in chemoreceptor and baroreceptor reflexes is probably due to the fact that the arterio-venous anastomoses in the pads which normally dominate the paw circulation do not participate in cardiovascular homeostatic reflex mechanisms but do respond vigorously when the regional vasomotor fibres are stimulated This suggestion is supported by the finding that after exclusion of the main part of the arterio-venous anastomoses from the paw circulation by clamping the pads the reflex skin vessel responses were the same as those obtained with direct stimulation of the regional vasomotor fibres

Even if the muscle capacitance vessel responses to chemoreceptor stimulation were relatively small compared with the concomitant muscle resistance vessel response there was in all cases a constriction of the veins This confirms earlier observations of a venoconstrictor response during chemoreceptor stimulation (Kahler Goldblatt and Braunwald 1962 Öberg 1964 Izuka *et al* 1970) although superficial cutaneous veins seem to dilate when the chemoreceptors are stimulated (Izuka *et al* 1970 Pelletier and Shepherd 1972)

No comparison between the renal vessel responses to chemoreceptor stimulation and regional vasomotor fibre stimulation was performed in the present experiments It is known however that lowering of baroreceptor activity leads to a less intense activation of the renal vasomotor fibres than of the skeletal muscle vasoconstrictor fibres (Kendrick *et al* 1972) As chemoreceptor stimulation increased renal vasomotor activity only slightly more than an unloading of the baroreceptors it is likely that the renal vasomotor fibres are also engaged less in chemoreceptor reflexes than those to the skeletal muscles

The present findings which suggest a more or less generalized increase of the sympathetic vasoconstrictor and accelerator fibre activity during chemoreceptor stimulation agree in many respects with earlier reported observations Bernthal and Schwind (1945) found a

reflex constriction in skeletal muscle and intestine in response to chemoreceptor stimulation. This constrictor reponse was subsequently found to be due to increased sympathetic vasoconstrictor fibre outflow (Bernthal *et al* 1945) Daly and Scott (1962) reported a reflex vasoconstriction in the limb skeletal muscle in the skin and in the mesenteric circulation when the chemoreceptors were stimulated in artificially ventilated dogs Öberg (1964) found that chemoreceptor stimulation in cats produced a reflex constriction of the resistance and capacitance vessels in skeletal muscle and intestine as well as a resetting of the pre/post capillary resistance ratio in the skeletal muscles leading to a capillary pressure fall and a net transcapillary fluid absorption in this tissue Stern and Rapaport (1967) observed an increased myocardial contractility a tachycardi and an elevated peripheral resistance as the immediate response to stimulation of the aortic chemoreceptors with nicotine in artificially ventilated dogs In contrast the latter authors found no consistent change of systemic flow resistance and an atropine sensitive bradycardia when the carotid chemoreceptors were stimulated with nicotine One should however probably be cautious when comparing the effects of hypoxic stimulation of the chemoreceptors with the responses to nicotine administration into the aortic and carotid sinus regions since with nicotine there may very well be a simultaneous stimulation also of other types of receptors in these areas and then preferentially non medullated endings (*cf* Paintal 1971) like *e g* non medullated baroreceptors (Sato Fidone and Eyzaguirre 1968)

The present observations differ also in other respects from earlier observations Calvelo *et al* (1970) found a reflex vasodilatation in the skin when the chemoreceptors were stimulated by nicotine or cyanide injected into the chemoreceptor regions in artificially ventilated dogs This dilatation was claimed not to be due to an inhibition of tonic sympathetic vasoconstrictor fibre activity nor to release of acetylcholine or other known vasodilator transmitter substances A vasodilatation in the skin in the rabbit s ear during hypoxia has been described by Zuntz (1878) and later by Chalmers and Korner (1966) and a reduced efferent sympathetic fibre activity in the rabbit s ear combined with an increased efferent activity in the splanchnic nerve has been reported during asphyxia by Iriki *et al* (1971) A reflex dilatation in cutaneous veins associated with constriction of hindlimb resistance vessels and splanchnic veins has also been reported to occur after chemoreceptor stimulation (Pelletier and Shepherd 1972) In contrast a clear reflex vasoconstriction (*e g* Chalmers and Korner 1966) and a significant increase of the efferent sympathetic activity in the rabbit s ear (Iriki *et al* 1971) occur in response to asphyxia if the sympathetic activity is first eliminated and the vessels dilated by means of a central heat stress It thus seems as if the vasodilatation in the skin during chemoreceptor stimulation generalized asphyxia or hypoxia affects mainly the arterio-venous anastomoses in the dog s pad and the rabbit s ear and the very superficial cutaneous veins in the dog which are directly involved in temperature regulation

In contrast to the findings by Calvelo *et al* (1970) vasodilatation in the skin was never observed during chemoreceptor stimulation in the present experiments This may be due to the fact that the temperature balance of the animals might have been such as to facilitate reflex constrictor responses according to the above mentioned findings by Chalmers and Korner (1966) and by Iriki *et al* (1971) Another and possibly more plausible

explanation is that in the present experiments no attempts were made to analyze more specifically the responses of arterio-venous anastomoses when the chemoreceptors were activated The experiments in which the paw vessel responses to chemoreceptor activation and regional vasomotor fibre stimulation were compared before and after exclusion of pad circulation suggest that no reflex *constriction* of the arterio-venous anastomoses in the pad was induced whether a reflex dilatation of these vessels occured cannot be resolved from the present experiments However the fact that essentially similar effects on paw circulation were produced by baroreceptor unloading and chemoreceptor stimulation does not support the idea that very potent specific vasodilator mechanisms are activated in just one of the two reflexes

Another observation in the present study which disagrees with earlier reported findings is that the heart rate increased when the chemoreceptors were stimulated This heart rate response occurred without delay upon arrival of the venous blood to the sinus regions and vanished rapidly in parallel with the vessel responses when arterial blood again reached the chemoreceptor region This positive chronotropic effect is therefore in all likelihood due to nervous rather than hormonal factors and must then be ascribed to an activation of the sympathetic cardio accelerator fibres since the animals were vagotomized In contrast Daly and Scott (1962) and Downing Remensnyder and Mitchell (1962) report an inhibition of the sympathetic influence on the heart in dogs during chemoreceptor stimulation In cats no change of impulse activity in efferent sympathetic fibres to the heart was observed during hypoxic stimulation of the carotid body chemoreceptors (Downing and Siegel 1963) Species differences may thus partly explain the discrepant observations

From the above discussion concerning the extent of engagement of various cardiovascular effector organs in chemoreceptor and baroreceptor reflexes the following general conclusions can be drawn Activation of the chemoreceptors as well as an unloading of the baroreceptors produces an equally strong reflex activation of the vasomotor fibres to the skeletal muscle and intestinal resistance vessels and in all likelihood to the nutritional skin vessels On the other hand the muscle capacitance vessels and the renal resistance vessels are definitely less engaged in chemoreceptor and baroreceptor reflexes than the muscle resistance vessels Finally the arterio-venous anastomoses in the pads do not seem to be engaged at all in cardiovascular homeostatic reflex mechanisms A comparison of the reflex influences from chemoreceptors and baroreceptors on the different vascular beds showed that the renal resistance and the muscle capacitance vessel responses were somewhat more pronounced when the chemoreceptors were stimulated than when baroreceptor activity was lowered The intestinal and skin vessels were evidently involved to the same extent in both reflex mechanisms

The background of the relatively moderate involvement of the renal resistance and muscle capacitance vessels in baroreceptor and chemoreceptor reflexes is not clear although it has been suggested that it may be due to a low spontaneous inherent activity in the central vasomotor neurons controlling the vasomotor outflow to these vessels (Kendrick *et al* 1972, Hadjiminas and Öberg 1968) This low spontaneous activity may in turn be explained by a higher excitation threshold of these neurons to various influences such as those emanating from higher central structures or those inherent in the prevailing

chemical composition of the immediate environment of the vasomotor neurons (Folkow Johansson and Lofving 1961) If the above suggestions were true one would expect that not only withdrawal of baroreceptor inhibition but also addition of chemoreceptor excitation should lead to less intense activations of the high threshold renal and capacitance vessel neurons This was indeed found in the present study However according to the present results the addition of chemoreceptor excitation seems to be slightly more effective in increasing the activity in these high threshold neurons than a withdrawal of baroreceptor restraint

This study was supported by grants from the Swedish Medical Research Council (B74 14X 644 10C) from the Medical Research Council London from Magnus Bergvalls Stiftelse and from Wilhelm and Martina Lundgrens Vetenskapsfond

Thanks are due to Mrs Gertrud Karlsson and Mrs Eva Cari Einarsson for skilful technical assistance

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Acta physiol scand 1975 93 52–58
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Some Metabolic Consequences of the Anaphylactic Reaction in the Rabbit

By

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Received 20 May 1974

Abstract

FREDHOLM B B and KJ STRANDBERG *Some metabolic consequences of the anaphylactic reaction in the rabbit* Acta physiol scand 1975 93 52–58

The administration of egg albumin to rabbits sensitized to this antigen caused marked increases in the arterial concentration of lactate glucose and glycerol but no change in the arterial FFA level Antigen administration had no effect in non sensitized rabbits Phentolamine (3 mg kg) or propranolol (1 mg kg) did not significantly alter the responses to egg albumin in sensitized rabbits Noradrenaline or sympathetic nerve stimulation decreased blood flow but caused no significant change in lipolysis in rabbit epigastric adipose tissue *in situ* It is therefore questionable if catecholamines are the major cause of the observed metabolic consequences of the anaphylactic reaction in the rabbit These metabolic events, *i e* increased lactate levels, lipolysis, and reesterification of fatty acids, are similar to those reported during hemorrhagic or endotoxin shock in dogs, in spite of species-differences and the difference in the genesis of the shock

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In hemorrhagic and endotoxin shock in dogs there is a marked elevation of arterial glycerol concentration indicating increased break-down of triglycerides (Spitzer *et al* 1973) This may be secondary to the increased sympathoadrenal activity (Chien 1967 Fedina *et al* 1973) since sympathetic nerve activity is a potent stimulator of lipolysis (cf Fredholm 1970).

On the other hand arterial FFA levels show little or no change in either type of shock (Kovách *et al* 1970 Spitzer *et al* 1973) Since the mobilization of FFA from the isolated subcutaneous adipose tissue is unchanged or even decreased during hemorrhagic shock in dogs (Kovách *et al* 1970) the authors have concluded that this is presumably due to an increased reesterification of fatty acids It is of interest that in the isolated perfused canine subcutaneous adipose tissue the degree of reesterification of fatty acids can be altered by a variety of experimental procedures (Fredholm 1970 1971 1972) including situations of increased (Fredholm 1974) or decreased (Fredholm and Karlsson 1970) oxygen delivery to the tissue In all these situations there is a correlation between the degree of re-esterification and the lactate pyruvate ratio (*e g* Fredholm 1971) Similarly in intact dogs there is a negative correlation between arterial lactate and FFA (Miller *et al* 1955) Arterial lactate levels

are known to be markedly elevated in hemorrhagic as well as in endotoxin shock in dogs (Chien 1967 Spitzer *et al* 1973)

Anaphylactic shock is an immediate hypersensitivity reaction characterized by severe and eventually fatal disturbances in ventilatory and circulatory functions In order to examine the possibility that the metabolic readjustments during shock show similarities in spite of species-differences and differences in pathogenesis we have followed the arterial concentration of lactate glucose FFA and glycerol in sensitized rabbits on antigen administration

Methods and materials

S n ti at on

Male alb no rabbits we gh ng 3 0–5 0 kg were given o er a period of 1_ days 10 intraperitoneal injections of 1 g egg albumin dissolved in 10 ml of saline solution The animals were used for experiments 6–8 weeks after sensitization Animals used as controls were housed in the same quarters but were gi en no injections.

Anaphylax

The animals were anesthetized with sodium pentobarbital (30 mg/kg) adm nistered i v A polyethylene catheter was inserted in the right femoral artery and further passed on to enter the abdom nal aorta This catheter was kept filled with heparinized saline solution Heparin was not administered systematically s nce it might inhibit the anaphylactic reaction (Kyes and Strauser 19_6) The catheter was used for blood sampling and for monitoring the s stem c blood pressure by means of a Statham pressure transducer (P 23 AC) The signals were recorded on a Grass model 5 B polygraph Anaph laxis was evoked by in ject ng 10 mg kg of egg albumin i v Control animals recei ed the same amount of egg album n Blood samples were drawn (see below) at times indicated in Fig 1 To some animals 3 mg kg of phentolam ne (Regitin® Ciba) or 1 mg kg of propranolol (Inderal® ICI) were given i v ?5 min before antigen admini stration At the end of the experiments a specimen of the ep gastric subcutaneous adipose tissue was excised for histamine analysis

Pe fused ep ga t ic adipo e t s e

In 4 rabbits the right epigastric ad pose t ssue was prepared fre of surrounding tissue accord ng to a mod fication of the method of Orö *et al* (1965) for dog adipose tissue as described by Lewis and Matthews (1968) Albino rabb ts we gh ng 3 0–4 _ kg were anesthetized with sod um pentobarbital (30 mg kg) The trachea and the left femoral artery were can nulated the latter to pe mit record ng of blood pressure by means of a Statham (P _3 AC) pressure transducer A sil con-filled drop-counter was inserted between the right femoral artery and the artery to the adipose tissue The venous outflow was directed into ice-cooled centrifuge tubes After centrifugat on aliquoted of arterial and enous plasma were analyzed for glycerol content In _ preparations a nerve located in a similar place as the nerve to dog adipose tissue (Orö *et al* 1965) was isolated, cut at the level of the inguinal canal placed on a bipolar silver electrode and stimulated (1–10 Hz, 10 V 2 ms for 3– 0 m n) In the two other preparations i a inject ons of noradrenal ne (0 5–50 µg) were gi en via a s de branch in the drop-counter

Chem cal d te m nat ons

Plasma or *n ri o* med um was used for the determ nation of glycerol (Laurell and Tibbl g 1966) FFA (Trout *et al* 1960) and glucose (GLOX reagent, KABI Stockholm) wh le blood was used for the deter minat on of lactate (TCC k t, Boehringer Mannheim)

Histamine was determined in adipose tissue and in incubates according to Shore *et al* (1959) The ad pose tissue (1 6–6 4 g) was minced and homogenized in 0 1 M HCl for 45 s us ng an Ultra Turrax homogen sator The tissue to acid ratio was 1 5

Results

Systemic responses

The rabbits used for the sudy were divided into 4 groups. Group I was comprised of non sensitized controls whereas the other animals were sensitized. Of the latter 3 groups one received no pharmacological pretreatment (Group II) while the other 2 groups were given phentolamine (Group III) and propranolol (Group IV) respectively.

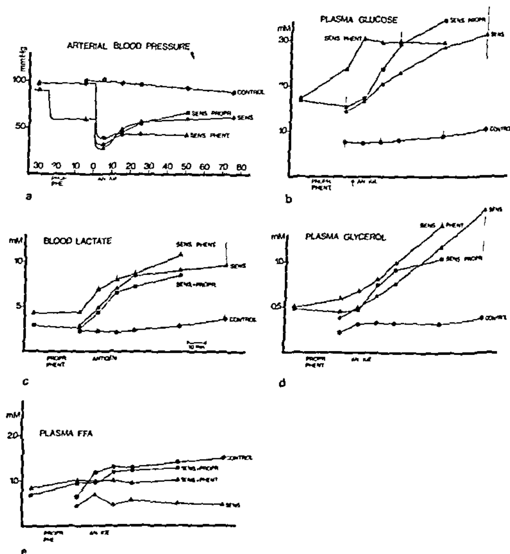


Fig. 1. The effect of egg albumin (antigen) administration in 4 groups of rabbits (mean ± S.E.). Group I (control) is non-sensitized animals (n = 8). Group II is sensitized rabbits having received no pharmacological pretreatment (n = 11). Groups III and IV are sensitized animals having received phentolamine (3 mg/kg) or propranolol (1 mg/kg), respectively 75 min prior to egg albumin (n = 8 and n = 5). *In all* groups an arterial blood sample was drawn 5 min before antigen. In addition a blood sample was drawn 30 min, *i.e.* 5 min before drug, prior to antigen administration in groups III and IV. Blood samples were also taken 5, 15, 25, 50 (all groups) and 75 (groups I and II only) after antigen administration.

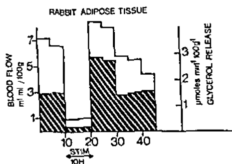


Fig 2 The effect of stimulation of nerve a (10 Hz 10 V 2 ms for 10 min) to isolated perfused rabbit epigastric adipose tissue prepared as described in Methods on blood flow (open) and glycerol release (striped) The rabbit weighed 3 ? kg and the isolated adipose tissue 6 5 g Hematocrit was 30 and arterial blood pressure 115 mm Hg

In Fig 1 is shown the effect of i v antigen administration blood pressure as well as arterial concentrations of glucose lactate glycerol and FFA in the 4 groups of rabbits The basal blood pressure (Fig 1 a) was virtually the same in all groups The administration of phentolamine resulted in a sustained fall in blood pressure with the consequence that these animals had a lower blood pressure than the others immediately before administration of egg albumin I v injection of egg albumin did not change the blood pressure in the control group None of the animals in groups I and IV died during the observation period In group II 3 out of 11 and in group III 2 of 8 animals died within 15 min

The basal arterial glucose concentration was significantly higher in groups II–IV than in controls (Fig 1 b) while there was no difference between groups in basal lactate level (Fig 1 c) Egg albumin injection did not significantly change the concentration of either metabolite in the control animals On the other hand both glucose and lactate concentrations showed a progressive increase in sensitized animals given the antigen

As with glucose basal arterial glycerol levels were higher in sensitized animals (Fig 1 d) Antigen had no effect on the glycerol level in control animals but caused a significant increase in the sensitized animals The data on plasma FFA were less consistent However there were no statistically significant differences between groups in basal FFA level (Fig 1 e) nor did antigen administration significantly alter the concentration of FFA in either of the groups

The histamine content was 1 43 ±0 27 µg/g tissue (mean ±S E) in subcutaneous adipose tissue from control animals (n = 8) and 1 03 +0 09 µg/g (n = 24) in tissue from sensitized animals having received antigen The difference was statistically significant ($p < 0\ 05$)

Perfused epigastric adipose tissue

In four rabbits the administration of noradrenaline (0 5–50 µg i a) or sympathetic nerve stimulation (1–10 Hz) decreased blood flow and the rate of glycerol mobilization After the constriction had faded a hyperemia was seen which was accompanied by an increased rate of glycerol release No net effect of the catecholamine or of nerve stimulation was however observed A typical experiment is shown in Fig 2 During the period of stimulation in this experiment there was a seven fold decrease in blood flow from 6 9 ml/min/100 g and a parallel decrease in the rate of glycerol release Relative to a control period of the same length 13 µmol/100 g tissue less glycerol was released during the stimulation period After

cessation of the stimulus blood flow returned to and above control level Simultaneously glycerol release rate increased Relative to a control period of the same length 13 5 μmol 100 g tissue more glycerol was released immediately after the stimulation period The net effect of the stimulation was therefore nil with regard to glycerol release

Discussion

In the present paper we report large increases in the arterial concentration of lactate, glucose and glycerol following the administration of antigen to sensitized rabbits The increase in lactate and glycerol is similar to that seen following hemorrhagic and endotoxin shock in dogs but neither of these conditions produce a hyperglycemia comparable to that observed in this study Whether this can be attributed to a species difference or to a difference between the different types of shock cannot be decided on the present evidence

Despite increased arterial levels of glycerol suggesting increased hydrolysis of triglycerides there was no change in arterial FFA levels following anaphylaxis in the rabbits The pattern of response is thus similar to that earlier reported in hemorrhagic and endotoxin shock in dogs (Rosell *et al* 1973 Spitzer *et al* 1973) It has been proposed that reesterification accounts for the observed difference in response of arterial glycerol and FFA to hemorrhagic shock in dogs (Rosell *et al* 1973) It seems possible that the response in rabbits has the same cause Thus in 3 different types of shock hemorrhagic endotoxin and anaphylactic there is an increased lactate level and evidence of increased lipolysis and esterification in spite of differences in genesis of the shock and in the species investigated

The metabolic effects observed in hemorrhagic and endotoxin shock in dogs are considered to reflect an increased sympathoadrenal activity (Chien 1967 Fedina *et al* 1973 Spitzer *et al* 1973) In contrast to the situation in dogs the adipokinetic effect of noradrenaline in rabbits is weak and inconsistent Rudman and co workers found no effect of 1 mg/kg noradrenaline s c while Svedmyr (1966) and Boberg *et al* (1970) reported increased plasma FFA levels during infusion of noradrenaline It is of interest that the latter authors found this effect only in rabbits weighing less than 2 5 kg Micheli (1969) found a negative correlation between lipolysis (as determined by glycerol release) and rabbit weight in perirenal rabbit adipose tissue (*in vitro*) In the present study rabbits weighing more than 3 kg were used This might explain the failure to note increased glycerol release from rabbit subcutaneous adipose tissue *in vitro* by 2 μM noradrenaline (unpublished experiments) Moreover sympathetic nerve stimulation (1–10 Hz) while causing pronounced vascular effects had no lipolytic effect Lewis and Matthews (1968) also failed to see any lipolytic effect of injected noradrenaline in the same preparation These results argue against the possibility that noradrenaline either released locally in adipose tissue or circulating is the primary cause of the increased plasma glycerol levels in sensitized rabbits given antigen Finally phentolamine or propranolol in doses that antagonized the cardiovascular effects of 5 μg of either noradrenaline or isoprenaline had no discernible effect on the metabolic response It cannot be excluded however that the doses of antagonists used in this study were too low to produce inhibitory effects since it is well established that systemically administered catecholamines are more effectively antagonized

that locally released The possibility that the observed metabolic effects are consequences of actions of other proposed mediators of anaphylaxis such as histamine (Schachter 1953) 5 hydroxytryptamine (Humprey and Jaques 1955) and slow reacting substance (Brockle hurst 1960) or even of the antigen antibody reaction itself must however be considered

Furthermore it is characteristic of shock irrespective of its genesis that tissue perfusion is decreased The consequent relative lack of oxygen leads to increasing lactate/pyruvate ratios As noted in the introduction there is a good correlation between this parameter and the degree of reesterification It is therefore possible that the increased lactate levels and increased re-esterification of fatty acids observed here on anaphylactic shock in rabbits and earlier in shock in dogs is a consequence of the shock condition rather than of the actions of the particular mediators of the symptoms

We thank Dr Kathryn Ballard for help with some of the experiments on perfused rabbit adipose tissue Mrs Birgitta Ask Miss Lotta Malmstrom and Miss Margareta Stensdotter for able technical assistance This study was supported by the Swedish Med cal Research Council (Grants no 40P 38 8 40X ?553)

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Acta physiol scand 1975 93 59-66
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Changes in the Fetal Heart Rate and ECG during Hypoxia

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Received 25 May 1974

Abstract

Rosén K G and I Kjellmer *Changes in the fetal heart rate and ECG during hypoxia* Acta physiol scand 1975 *93* 59-66

Previous reports on the fetal hypoxic bradycardia in animals have indicated that there is a vagal influence especially when asphyxia is induced by umbilical cord occlusion In the present study hypoxia was induced via the mother thus keeping the fetal circulation intact The experiments were conducted on mature fetuses of three different species namely 20 guinea pig, 3 cat and 3 lamb fetuses The ECG was recorded continuously and used for measuring the fetal heart rate The vagal influence on the fetal hypoxic bradycardia was tested by comparing the time for the onset of the bradycardia with or without vagal activity There was no indication of any vagal component in the fetal hypoxic bradycardia which is therefore to be regarded rather as a sign of myocardial hypoxia and failing fetal circulation The ECG recordings showed that the fetal bradycardia initially is an AV block type II and that there are progressive changes in the S-T interval as an early sign of hypoxia

Continuous fetal heart rate (FHR) monitoring is today the most used way to study the fetal situation during labour Clinical investigations by *e g* Caldeyro-Barcia *et al* (1966) and Hon (1959) have shown that when the fetus reacts with intense bradycardia this response is well correlated to the degree of fetal hypoxia and acidosis It is however remarkable that in spite of its great clinical importance the mechanism behind this fetal bradycardia is still incompletely known

Several studies on the vagal influence on FHR in different animals have been performed Thus Barcroft (1946) demonstrated that the fetal lamb reacted with a rapidly developing, vagally mediated bradycardia during occlusion of the umbilical cord The mechanism behind was supposed to be a baroreceptor reflex because of the increase in arterial blood pressure associated with the bradycardia After bilateral vagotomy the lamb fetus reacted with a more slowly developing anoxic type of bradycardia. By selective occlusions of the umbilical artery and vein in the lamb fetus Reynolds (1954) showed that occlusion of the umbilical vein led to a rapidly developing bradycardia in spite of a decrease in blood pressure while he observed the same response as Barcroft (1946) on selective occlusion of the umbilical artery The findings were essentially the same after bilateral

vagotomy though with a more delayed slowing of the heart in connection with the development of anoxia

Fetal rabbits were studied by Bauer (1938) observing a non vagal bradycardia during umbilical cord occlusion and that asphyxia led to vagal bradycardia first when the newborn rabbit was about 10 days of age This would indicate that the degree of neurological maturity is one factor which could explain the difference in vagal activity

The results of Barcroft (1946) and Reynolds (1954) mentioned above indicate that umbilical cord occlusion with interruption of the circulation over the placenta which receives over 50 per cent of cardiac output leads to extensive redistributions of blood flow to other vascular beds Accordingly marked changes in arterial and venous pressures are also induced activating arterial venous and/or cardiac mechanoreceptors leading to a reflex in which vagal bradycardia is one component Therefore the umbilical circulation must be kept intact in case adequate studies on the fetal circulatory regulation during graded hypoxia are to be performed Moreover during umbilical cord occlusion hypoxia is inevitably accompanied by an increase in the CO_2 tension of the fetal blood This means that hypoxia has to be induced *via* the mother for instance by letting her breathe a gas mixture with a low oxygen concentration This approach was used by Reynolds (1958) in fetal lambs showing that the fetus exhibited an increased tendency to react with bradycardia when the ewe was given gas mixtures with low oxygen tension and atropine given during the bradycardia resulted in some increase in FHR One mechanism behind such a vagal hypoxic bradycardia could be a stimulation of left ventricular distension receptors studied by Thorén (1972) in adult cats These receptors are activated also by severe asphyxia causing ventricular dilatation leading to reflex vagal bradycardia among other things

The purpose of the present investigation was to explore in more detail to what extent vagal activation contributed to the fetal bradycardia when hypoxia was induced during *intact umbilical circulation*

Methods

The experiments were conducted on 3 different animal species namely guinea pig, lamb and cat fetuses

18 pregnant guinea pigs with 0 fetuses were the main experimental material The gestational age ranged from 55 to 64 days (term about 65 days) This was estimated from the weight of the mother (over 1 100 g) and the mean fetal weight, using standard curves from Draper (19 0)

The neurological maturity could roughly be tested by watching the strong fetal movements as a response to mechanical stimulation

The guinea pig mother was anesthetized with ether as an induction and then tracheotomized After cannulation of a carotid artery anesthesia was maintained with urethane (00 mg/ml) in small doses (at a total of 1.0 g/kg) Maternal blood pressure was measured from one carotid artery and recorded on a Grass polygraph 7 A recorder by means of a Statham pressure transducer From the same artery blood gas samples were taken during the hypoxic periods.

The fetus was taken out by a midline cesarean incision placed on a disc and immediately covered with pads soaked in warm saline The temperature was kept at about 38 C by radiation. To prevent the fetus from breathing a rubber bag was placed firmly over its nose

During the entire experiment great care was taken to prevent tension upon the umbilical cord

Both vagal nerves were dissected free and in most experiments placed on small probes which could be cooled down to 0 C by perfusing them with a mixture of ice water and alcohol In this way reversible vagal block could be induced. In some cases bilateral cervical vagotomy was performed instead *In only a few*

experiments it was possible to cannulate the right carotid artery allowing a direct recording of fetal blood pressure

The fetal heart rate was monitored from the ECG which was recorded as a precordial lead with crocodile clips placed on the left part of the chest and on both right legs Th ECG impulses were recorded on a Grass polygraph 7 A recorder

The guinea pig mother was artificially ventilated throughout the experiment Blood gas analyses were made in order to control the respiratory status Gas mixtures with an oxygen concentration of 3 to 10 per cent were given to the mother *via* a ventilator The ECG was recorded every 15 s during a period of 5 s After 60 to 90 s of hypoxia arterial blood gas samples were taken and analyses for blood gas tensions and pH were immediately made at 38 C with a Radiometer pHM27GM using standard P_O and P_{CO} electrodes

The hypoxic period was stopped after 3 to 5 min or when there was a marked decrease in FHR A control period of 10–15 min was inserted between the hypoxic periods The fetuses were randomly exposed to hypoxia with or without vagal block

Parallel experiments were performed also on 3 mature cat fetuses and 3 lamb fetuses (120–130 days of gestation term at 145 150 days) The anesthesia and preparation methods of the lamb experiments have been described in a previou report by Kjellmer *et al* (1974) ECG recordings were also made during the lamb experiments In the cat and lamb experiments FHR was measured on the Polygraph recorder by a tachograph that was triggered by the arterial pressure

Results

The duration of the hypoxic periods varied between 3 to 5 min and hypoxia was stopped when a marked bradycardia could be seen which was usually quite sudden in onset rather than gradual

A total number of 23 equivalent hypoxic periods were recorded with or without intact vagal control The vagal effect on the fetal hypoxic bradycardia was tested by comparing the time for the onset of the bradycardia when the vagal nerves were intact or not The maximal range on time difference was 45 s and when calculating the mean time difference there was no difference to be seen neither was there any increased reduction of the FHR with or without intact vagal control

Maternal arterial blood gas analysis were made in order to measure the extent of fetal hypoxic stress However when comparing the tim^ for the onset of fetal bradycardia with the maternal blood gas values there was no regular correlation between them

The ECG recordings then provided more information than the mer recording of heart rate alone and the different ECG components could usually be clearly identified Fig 1 shows an example of a moderate hypoxic period recorded in a mature guinea pig fetu There is a slight decrease in heart rate and blood pressure but no more intense bradycardia was obtained However progressive changes in the ECG were quite obvious in the form of an increased T wave amplitude and an elevation of the S–T segment the latter phenomenon being regularly observed In some experiments negative T wave changes could be recorded instead As can be seen in Fig 1 these ECG changes are normalized during the recovery period

Fig 2 shows ECG recordings from three guinea pig fetuses where the abrupt onset of bradycardia is obvious The figure demonstrates that the fetuses reacted with an AV block type II during the initial phase of bradycardia The hypoxic T wave changes are also demonstrated No differences were observed in the occurrence of these ECG changes when vagotomy was performed Dropped beats could be seen as an early sign of hypoxia

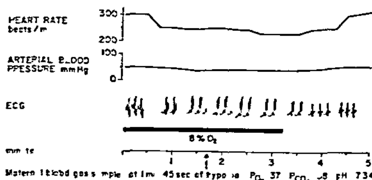
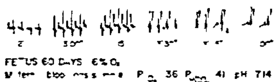
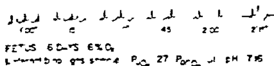


Fig. 1 Effect of moderate hypoxia in a mature guinea pig fetus. Note the progressive changes in the configuration of the ECG

As previously stated, the hypoxic S–T elevation was a constant finding, and in Fig. 3 some examples of the time relationship between the onset of these changes and the fetal bradycardia are shown. In moderate degrees of hypoxia, (*i.e.* pH $\geq$ 7.26 and $PaO_2 \geq 30$ mm Hg) no bradycardia occurred in spite of marked changes in the S–T interval. When the fetal hypoxic stress was increased at still more lowered maternal pH and PaO_2 the S–T changes remained an earlier sign of fetal hypoxia than bradycardia. In some cases hypoxic S-T changes could be seen already at the beginning of the hypoxic period. These fetuses had previously been exposed to a hypoxic period.

In those cases where arterial blood pressure could be measured (10 hypoxic periods), the *fetus reacted with a marked pressure fall in association with the bradycardia.* No consistent changes in blood pressure could be obtained when only S–T changes were recorded. In Fig. 4 recordings in a mature lamb fetus during grave hypoxia $(7\% \ O_2)$ is shown, still without any signs of a vagally mediated bradycardia. Instead it seems as if the vagal blockade leads to a fall in blood pressure in association with a bradycardia response. With





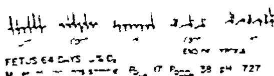


Fig. [illegible] Recordings of the abrupt onset of the bradycardia in three guinea-pig fetuses. The AV-block type II is demonstrated [illegible] with hypoxic T-wave changes.

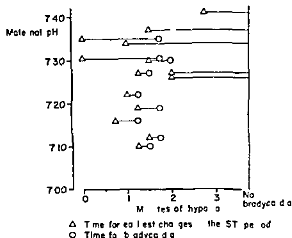


Fig 3 Figure showing the time relation ship between the onset of the hypoxic S-T changes and the fetal bradycardia compared with the maternal pH values

intact vagal function the fetus reacts with initial increases in blood pressure and heart rate followed by a gradual decrease in blood pressure here associated with a tachycardia which eventually turns abruptly into a marked bradycardia Also in the lamb experiments the hypoxic S-T changes can be easily and clearly recorded AV block type II has also been recorded during the initial bradycardia

3 pregnant cats were also investigated Fig 5 shows one experiment where the fetuses after preparation were replaced in the uterus The carotid artery was cannulated and blood pressure and heart rate were recorded In fetus I the vagal nerves were kept intact while they were cut in fetus II *Simultaneously with the onset of bradycardia there*

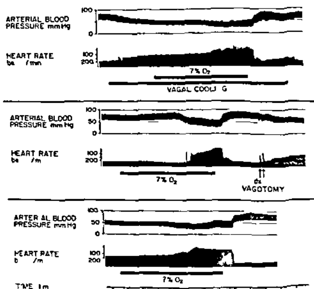


Fig 4 Effect of grave hypoxia in a mature lamb fetus with or w th out vagal acti ity Note the agal effect on the FHR and blood pres sure during the initial phase of the hypox c periods

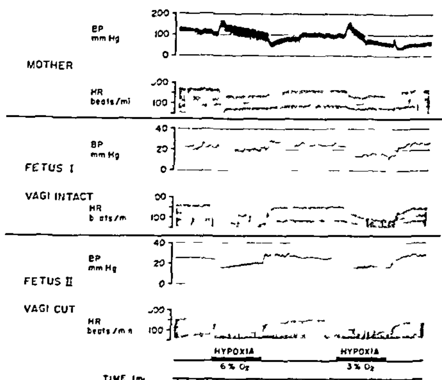


Fig. 5 Effect of hypoxia during intrauterine conditions in two cat fetuses In fetus I the vagal nerves were kept intact while in fetus II they were cut.

is a fall in blood pressure which then is kept at a fairly constant level until the mother is given air for breathing. No vagally mediated contribution to the bradycardia response to hypoxia could be seen The bradycardia was here more marked and appeared about 30 s earlier during 3% O_2 hypoxia compared with 6% O_2

Discussion

The main purpose of the present study was to test whether maternally induced fetal hypoxia gives rise to any vagally mediated bradycardia or not For these purposes three different species of animals were investigated Both the lamb and the guinea pig fetus can be regarded as neurologically mature while the cat fetus is in this respect more immature at term The degree of neurological maturity is one factor that may strongly influence the fetal circulatory response to hypoxic stress However in none of these species were there any signs of a vagally mediated component in the bradycardia response elicited by a maternally induced hypoxia It is on the other hand clearly demonstrated in earlier studies (Barcroft 1947 Reynolds 1954) that the lamb fetus has an ability to react with an increased vagal activity leading to a bradycardia when hypoxia is induced by umbilical cord occlusion Thus the degree of neurological maturity could not explain why no vagal bradycardia was observed in the present experiments

It has previously been stated by Sholander (1960) that the fetal bradycardia may be

considered as a form of a diving reflex This reflex is among other things a vagal reflex in association with intense neurogenic vasoconstriction which enables diving animals like seals to reduce and redistribute their cardiac output to favour only the vitally most important organs the heart and the brain leading to the most beneficial utilization of the large quantity of oxygen stored in the lungs and the blood

However the fetus has no such oxygen stores available and must instead utilize anaerobic glycolysis as its main energy source It has been demonstrated by Dawes *et al* (1963) that a correlation exists between survival time and myocardial glycogen stores and it has also been reported by Su and Friedman (1973) that the fetal heart is more capable of and dependent on glycolytic than aerobic metabolism during hypoxia as compared to the adult heart Thus there is not the same need for oxygen conserving reflex adjustments in the fetus where a high blood flow to the vital organs may be more important in terms of eliminating anaerobic waste products like lactate If so the non vagal fetal bradycardia should be a sign of myocardial hypoxia and failing fetal circulation Thus no vagal component could be recorded in any of the three species of animals investigated and it seems as if the vagal bradycardia previously recorded during umbilical cord occlusion rather implies a reflex brought about by haemodynamic changes and not by hypoxia alone

Activation of the left ventricular distension receptors could be one mechanism behind this type of bradycardia but they do not seem to be activated when hypoxia is induced *via* the mother and the fetal circulation kept intact

The lamb experiments suggest the presence of vagal afferent impulses blocked by cervical vagotomy which induced stimulation of the vasomotor center during grave hypoxia resulting in reflex tachycardia and increased blood pressure Activated aortic chemoreceptors are likely to be the cause of this effect

The fetal hypoxic bradycardia was as mentioned essentially the result of AV block type II The mechanism behind this could be that the atrial myocardium has a higher tolerance against hypoxic stress than the ventricular myocardium Su and Friedman (1973) showed that an isolated spontaneously beating SA node right atrial strip had a greater ability to withstand profound hypoxia compared with a ventricular myocardial strip tested under the same conditions for generating contractile force The AV block could also be explained by a low hypoxic tolerance of the AV node

The continuous fetal ECG recordings made during the experiments gave also another interesting item of information namely the progressive hypoxic changes in the S–T interval occurring well in advance of any bradycardia and fairly well reflecting the degree of hypoxia The S–T interval represents the repolarization of the myocardium which is regarded as an active process T wave variations may be of two basic types The first type depends upon accompanying variations in the sequence of ventricular depolarization represented in the QRS complex The other one occurs independently of QRS and is mainly the result of haemodynamic metabolic electrolyte or other functional abnormalities The hypoxic S–T changes are to be assigned to this latter group and judging from the very marked increase in T wave amplitude a rise in plasma potassium may be one of the underlying mechanisms Since the pattern of ECG changes was unaffected by vagotomy a vagal component of these changes can be ruled out

In conclusion

This investigation on guinea pig lamb and cat fetuses has shown that *no* sign of any vagal component was present in the bradycardia response to hypoxia when induced *via* the mother thus leaving the umbilical cord intact

Continuous fetal ECG recordings during hypoxia showed that the fetal bradycardia, usually abrupt in onset is due to an AV block type II and that there are progressive changes in the S-T interval which can serve as the most early sign of hypoxia in the fetus

This study was supported by a grant from the Medical Faculty Göteborg and from the Swedish Medical Research Council No 19X 2591

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Conductance Recording of Ionic Outflow from Frog Skin Glands during Nerve Stimulation

By

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Received 29 May 1974

Abstract

LANG L E SJOBERG and C R SKOGLUND *Conductance recording of ionic outflow from frog skin glands during nerve stimulation* Acta physiol scand 1975 *93* 67–76

A method for cont uous record ng of the ionic efflux from frog sk n glands has been developed which under given e perimental conditions provides a con enient index on glandular secretion A nerve skin preparation from the calf is mounted so that the outside of the skin forms the bottom of a small test com partment with distilled water while the corium s de is bathing in Ringer s solution After determination of the adequate nerve stimulus parameters for discernible gland secretion to occur the skin nerve was stimulated at 10 Hz dur ng varying periods and chemical control analyses performed of the changes in ionic content of the test compartment solution The main ionic outflow consisted of Na^+ and Cl^- in equal amounts the outflow of other ions during stimulation being negligible The concom tant conductance changes were measured as variations of absorption in a high frequency field applied to a conductance probe placed in the test chamber Determinations of the NaCl outflow based on the conductance changes were in good agreement with the values obtained by chemical analysis The continuous conductance recordings proved to gi e approximative information of the quantities and time relat ons of the glandular secretion allowing a direct comparison with *e g* skin potent al changes

There are two major types of skin glands in frog, the mucous glands producing a watery secretion without any formed elements and the granular glands with a more dense secretion containing light reflecting granulae The smaller mucous glands are found in a large number all over the body whereas the larger granular glands are more scarce and situated mostly on the dorsal parts of the body and also along the edges of the toes

The function of the skin glands in the permeability control of frog skin was early recog nized by Koefoed Johnsen *et al* (1953) Several investigations have since been performed in order to illuminate this question and also to analyze the secretion processes as such In one type of investigations the glands were stimulated by application of neurohormones either subcutaneously in intact animals (Campbell *et al* 1967 Friedman *et al* 1967 Wat lington and Huf 1971) or to the corium side of excised skin preparations (*e g* Seldin and Hoshiko 1966 McAfee 1970) Analyses of the action of catecholamines as well as of adren ergic blocking agents showed that a β adrenergic mechanism is involved in the secretion

processes Determinations of the chemical composition of frog gland secretions showed that Na^+ and Cl^- are the dominant ions in the mucous efflux

Investigations of another type in which nerve stimulation was used for activation of the glands are relatively few Schoeffeniels and Salee (1965) reinvestigated the phenomenon of skin potential change occurring during cutaneous nerve stimulation originally described about a hundred years ago (*e g* Engelmann 1872) They interpreted the observed potential change as being due in the first place to a nervous influence on the non glandular epithelium but the possibility of gland activation was also taken into account Lindley (1969) studied the effect of skin nerve stimulation on frog skin by measuring different parameters such as electromotive force and short circuit current and attributed the dominant changes thus induced to glandular activity As pointed out by House (1969 1971) who made extensive permeability studies of frog skin under varying experimental conditions the interpretation of the results is rendered somewhat difficult by the lack of a simple and reliable index of glandular secretion

It occurred to us that a method earlier used in studies of the ionic outflow from algae cells (Haapanen and Skoglund 1967) might be applicable in studies of this type As will be shown in the following this method based on conductivity measurements can under certain given experimental conditions give information about the quantities and time relations of the ionic outflow from the skin glands and thus serve as a suitable index of the secretion allowing a direct comparison with *e g* skin potential changes

A neurophysiological analysis of the sympathetic fiber group involved in the gland secretion will also be presented

A preliminary report of these results was given at the XIV Scandinavian Physiological Congress (Lang Sjoberg and Skoglund 1973)

j

Methods

Material Frogs of the species *Rana temporaria* were used in all experiments A skin area of the calf as chosen for these stud es because of its homogeneous content of mucous glands with very few if any granular glands The mucous glands are situated close to each other in a number of about 50 glands per mm^2 skin area The indi idual gland measures up to 100 μ in diameter and is composed of an inner layer of cuboidal cells facing a central duct and an outer layer of myofibrillar sheath cells

Expe imental set p A piece of calf skin of about 2 cm^2 area was dissected out with the attached cutaneous branch of the sural nerve togeth r with the sciatic nerve trunk The skin was mounted in a Perspex chamber (Fig 1) with the corium side downwards making contact with the Ringer s solution in the lower compartment The outer surface of the skin forms the bottom (0 63 cm area) of the upper smaller te t compartment which has a olume of 0 5 ml and is filled with distilled water at the beginning of the experiment Before this started the compartment was rinsed several times with distilled water to remo e impurities A conductivity probe consisting of thin platinum electrodes is placed close to the outer skin surface Between the two electrodes a h gh frequency field (1 095 kHz) is appl ed from a high frequency reflectometer of Haapanen s design (196) The application of this technique for mea urements of minute changes in ele trolytic conductance has been descr bed in detail earl er (Haapanen 1967 Haapanen and Skoglund 1967) and only the basic principle will be referred to here a change in conducti ity occurr ng in the fluid between the two electrodes will be reflected a variations in absorption of the h gh frequency field and th s is recorded by the refle tometer as changes in its grid current alue (I_g) which can be read on an ammeter and also continuously recorded on an inkwriter The probe reflectometer is cal brated again t solutions of known ionic content and the result plotted in a d agram showing the relati n between I_g a d

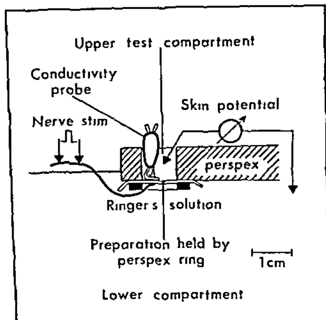


Fig. 1 Schemat c draw ng of the two-compartment Perspex chamber used for conductance measurements See text

ionic concentration Such a diagram for NaCl solutions actual for the present investigations is shown in Fig. 2

Ch m cal anal s Samples from the test chamber were analyzed by flame photometry (Beckman DU2 with recorder) for sodium potassium and calcium Chlor de ions were determined by a potentiometric method

St m lation and rec ding a angements A Grass S4 stim lator was connected via an isolat on unit to the stimulus electrodes appl ed 5 mm apart to the sciat c nerve trunk wh ch was lifted out of the Ringer s solution during stimulation The change in I_g values of the reflectometer was continuously recorded on one channel of a Grass polygraph Another channel was used for record ng of the potential changes across the sk n measu ed by calomel electrodes placed in each of the two compartments and connected to a Grass P6 preamplifier Con entional apparatus was used for the nerve recordings

Results

Properties of sympathetic fibers involved in glandular secretion

In view of the controversial data found in earlier publications with regard to the stimuli suitable for nervous activation of the glands it seemed pertinent to make a special study to determine the adequate stimulus parameters of the sympathetic fibers involved and al o to try to classify these fibers The formation of small droplets at the mouths of the glands easily visible in a binocular dissecting microscope was used as index of glandular activa tion When the stimulus electrodes were applied to the sciatic nerve the threshold stimuli were found to be fairly constant on repeated stimulations *in one and the same experiment* and also in different experiments In this nerve trunk the sympathetic fibers are obviously better protected against damaging factors such as drying during the stimulation periods

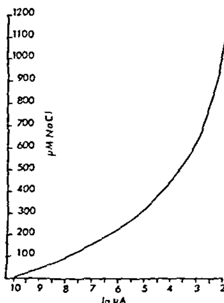


Fig. 2. Relation between I_g values as indicated on the reflectometer (*abscissa*) and NaCl molarity (*ordinate*) at 20 C

than if they had been exposed for stimulation at the sympathetic chain On the basis of strength-duration determinations 3 ms was chosen as a suitable standard stimulus duration. The average stimulus strength was then found to be about 4 V only occasionally was secretion observed at 3 V or was a stimulus of 5 V necessary These values represented about 10 times the stimulus strength required for excitation of the fastest fibers of the A group

On the basis of these threshold determinations for discernible gland secretion it was then possible to choose the adequate stimuli for the main series of experiments in which conductance changes were used as index of secretion In these often longlasting experiments it was above all of importance to avoid unnecessarily strong stimuli which might cause stimulus threshold variations due to nerve damage

The knowledge of the threshold stimulus value of the sympathetic fibers engaged in glandular secretion made it also possible to classify these fibers by determining the conduction speed of the sympathetic nerve volley which is elicited in response to that particular stimulus strength For this purpose the sympathetic chain was exposed for recording *in situ* and the stimulating electrodes placed on the skin nerve to the calf The result of such a recording is shown in Fig. 3 (A) which for comparison also shows the compound action potential recorded from the sciatic (B) A series of five experiments showed that the fibers belonged to the group having a conduction speed of 2–4 m/s which corresponds to the fast group of sympathetic skin fibers described by Loewenstein (1956)

Chemical determinations of ionic outflow during rest and activity

Several analyses have been made of the chemical composition of the skin secretion evoked by catecholamine injections in intact frogs (Campbell *et al* 1967 Friedman *et al* 1967 Watlington and Huf 1971)

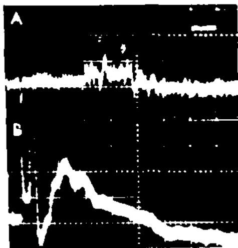


Fig 3 *A* Antidromic nerve volley recorded in postganglionic sympathetic fibers in response to stimulation of skin nerve to calf conduction distance 40 mm conduction speed 3–4 m s, stimulus duration 3 ms stimulus strength 4 V *B* Response of whole skin nerve to the same stimulus Time 5 ms

It is generally agreed that this secretion which can be assumed to be primarily mucous gland in origin contains Na^+ as the dominant cation whereas K^+ ions do not occur in larger amounts than in the resting outflow Cl constitutes the dominant anion in the catecholamine induced secretion but there may also be an increased outflow of HCO_3 ions as part of a pH regulating mechanism in the skin In a study of the effect of catecholamines on the outflow from isolated frog skin with radioisotope technique McAfee (1970) could show that Na^+ and Cl^- ions occur in equal amounts

The only previous data on ionic content of skin secretion after nerve stimulation seem to be those from one experiment on isolated skin from the axillar region (Schoeffeniels 1967) which showed that there is a large increase in outflow of Na ions but only a small increase in K^+ outflow as against that in resting secretion the relation between the two ions in the nerve induced secretion during stimulation being about 20 1

Results essentially similar to those described above were obtained in a series of chemical analyses of our calf skin preparation which were made to serve as a basis for interpreting the conductance changes

Flame photometric analyses of Na^+ K^+ and Ca^2 in samples from the test compartment taken after various periods of non stimulation show that in addition to Na^+ ions K^+ and Ca^2 may occur in various amounts but always in lesser concentrations than Na^+ (from zero up to maximally 20–30% of the Na^+ concentration) which itself is kept at a low level in comparison with that during active secretion (*cf* below)

In agreement with the results from catecholamine activation no significant increase in the outflow of K could however be demonstrated during nerve stimulation the dominant changes being those of Na and Cl^- ions

Data obtained from a comparative analysis of the outflow of Na^+ and Cl^- during nerve stimulation and non stimulation periods in a typical experiment are given in Table I It appears that the resting outflow is negligible in relation to that occurring during glandular activation This result seems to indicate that the unusually large concentration gradient

TABLE I Comparison between Na^+ and Cl^- concentrations in μM of test compartment fluid (ohm 0.5 ml) after varying periods of rest and glandular activation

	Test period in s	Na^+ during whole period	Na^+ per 10 s	Cl^- during whole period	Cl^- per 10 s
Non-stimulation period	300	22	0.7	21	0.7
	4 500	40	0.09	36	0.08
	6 000	94	0.15	80	0.13
Nerve stimulation period	20	32	16	41	20
	40	81	20	84	21
	60	56	9	60	10

which is set up across the skin due to the Ringer's solution on the corium side and the distilled water initially applied on the outer side has not caused any strikingly large resting outflow from the (glandular or non glandular) epithelium. As shown by chemical analyses as well as by conductance measurements there may occur a more rapid outflow of ions immediately following application of distilled water, but after a few minutes a stabilization takes place to a relatively slow flow rate which may either be negligible in relation to the outflow during activation, as shown in Table I, or else so constant that its size can be calculated over a certain period of time. It also appears from Table I that there is a strikingly good correspondence between Na^+ and Cl^- ionic outflow both during the non stimulation and the stimulation periods, respectively.

The chemical analysis has thus shown that these two ions constitute the dominant outflow in the glandular secretion from excised calf skin preparation during nerve stimulation and *can be assumed to be the main cause of the conductance changes that can be recorded* (see below). Further chemical determinations of the test compartment fluid parallel with conductance measurements will show this to be a reasonable assumption.

Conductance measurements

A typical recording of the conductance change taking place as a result of the increase in ionic outflow from the glands during nerve stimulation is shown in the upper curve of Fig. 4 (in which the lower curve illustrates the concomitant changes in skin potential, cf. below). After a latency of a few seconds the nerve stimulation results in a marked upward deviation of the baseline lasting throughout the stimulus period of 30 s. This phase of increased conductance reflects the increase in ionic concentration occurring close to the surface layer of the skin where the probe is placed. After the end of the secretion period there occurs, as a result of diffusion in the test compartment fluid, a return to a somewhat lower conductance value which is then maintained. This new level of increased conductance corresponds to an I_g value of 5.3. On the assumption that the conductance is solely due to the presence of Na and K ions this value would correspond to a NaCl concentration of 300 μM (*cf.* diagram in Fig. 2). In this case a flame photometric analysis of the Na^+ ions concentration in the compartment fluid was also made and gave as result 294 μM Na

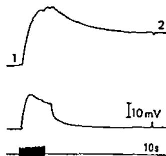


Fig 4 *Uppe cu re* Typical course of conductance change recorded as changes in I_g value of the reflectometer probe in test chamber as result of nerve stimulation Initial I_g value (at 1) 5 9 correspond ing to 300 μM NaCl final I_g value (at 2) 5 3 corresponding to 240 μM NaCl resulting in an approximate net outflow of 60 μM NaCl *Lower curve* concomitant change in potential level recorded across the skin *Bottom line* stimulus indication

Cl ions were not determined in this particular experiment but on the basis of previous analyses an equal outflow of these ions can be assumed and there is thus a strikingly good correspondence between the results of the conductance method and the chemical analysis in this experiment In view of the sources of error involved in both methods it is obvious that these errors have cancelled each other in this case However even if the actual difference was somewhat greater (*cf* below) the result definitely supports the conclusion that the conductance change is mainly due to NaCl content For obvious reasons it is not possible to take out the fluid in the test compartment for chemical analysis before stimulation but if one assumes that NaCl accounts for the main conductance recorded prior to nerve stimulation corresponding to an I_g value of 5 9 this would imply that the NaCl concentration then was 240 μM The conclusion can thus be drawn that there has been an increase in NaCl concentration of 60 μM Knowing the volume of the compartment fluid this value can easily be translated to weight NaCl and was found to be 1 85 μg NaCl

Usually there is a somewhat greater discrepancy between the concentration values obtained by the two methods as appears from Table II When occasionally as in expt 4 the conductance method gives a lower value of ionic concentration than the chemical analysis this is obviously due to methodological errors In most cases the conductance value represented a somewhat higher ionic concentration than that accounted for by the NaCl concentration as obtained by the chemical analysis This might indicate the presence of other ions Flame photometric analyses show that in the first place K^+ but to a certain

TABLE II Comparison between values of Na^+ concent ation in μM of test compartment fluid (volume 0 5 ml) obtained by conductance measurement and flame photometry

	Experiment						
	1		3	4	5	6	7
Conductance measurement							
I_g	8 5	7 8	7 8	7 2	6 8	5 6	5 3
Na	66	106	106	145	173	254	300
Flame photometry							
Na	60	9	8	174	174	240	294

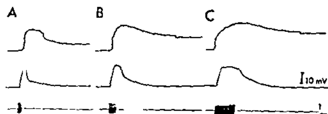


Fig 5 Conductance changes (*upper records*) in comparison with skin potential changes (*lower records*) during increasing stimulus durations indicated by stimulus markings below Stimulus durations and the resulting increases in concentration expressed in μM NaCl respectively in *A* 5 s 24 μM in *B* 10 s 48 μM and in *C* 30 s 72 μM

extent also Ca^{2+} might be the cations contributing to the high conductance value but anions like SO_4^{2-} and HCO_3^- might of course be involved as well However from comparative analyses of resting secretions it seems likely that the K^+ and Ca^{2+} ions emanate from a spontaneous leakage of the skin prior to the active secretion from the glands To judge from our experience it does not seem to imply any greater error if one assumes that the *difference* in conductance before and after the nerve stimulation is to be interpreted as an increase solely in NaCl concentration The error thus introduced can be estimated to be maximally 5%

On a second channel the potential across the skin was routinely recorded in the first place as a check on the state of the preparation deterioration being reflected as a change in resting potential level In a forthcoming study the change in skin potential during nerve stimulation will be taken up for closer analysis and here we only want to show the typical monophasic change in positive direction which occurs under the given experimental conditions with a low ionic concentration on the outside of the skin (*cf* Schoeffeniels and Salee 1965)

Fig 5 serves to illustrate the applicability of the conductance method as a simple quantitative index on glandular secretion In this experiment the stimulus durations were varied, and the records show how the ionic outflow increases with increasing stimulus durations. The simultaneously recorded skin potential changes show a certain similarity to the conductance recordings We do not want to draw any premature conclusions from this relationship but obviously such a dynamic recording of the ionic secretion parallel with the potential recording might throw further light on the electrical skin processes

Discussion

The aim of the present work has been to develop a method of direct recording of the net ionic outflow from frog skin a method sensitive enough to record the glandular outflow from a comparatively restricted area of the skin during relatively short periods of nerve stimulation We have chosen this type of stimulation *i e* because it could be assumed to exert a more selective effect on the glandular epithelium than would application of neurohormones which may affect the non glandular epithelium as well The possibility mentioned in the introduction that the latter is also under nervous control cannot be excluded but

preliminary studies of the innervation of the calf skin using fluorescence technique have so far not given any positive support for a sympathetic innervation of the non glandular epithelium (Sjoberg unpublished observations) Needless to say the conductance method of recording the ionic flow is applicable also in experiments with neurohormonal stimulation

The conductance method has the obvious advantage that it is possible directly to follow the events during an acute experiment In connection with the presentation of the results we have pointed out the possible disadvantage of keeping a low outer concentration necessary for attaining the desired sensitivity of the method However the working range of the reflectometer of up to 1 200 μM NaCl implies that the experiments can be performed under outer concentrations that are not too unphysiological considering that the frogs are kept in tap water before the experiment To judge from our experience so far the active glandular secretion se ms to be fairly independent of the ionic concentration of the outer medium

The properties of the probe/reflectometer unit have been discussed in detail in previous papers (Haapanen 1967 Haapanen and Skoglund 1967) We would only like to emphasize here that the double-electrode probe has been shown to provide a sufficient shielding effect against conductance changes occurring *within* the frog skin during the glandular activation The sensitivity of the probe is thus restricted to conductance changes occurring in the gap between the electrodes This implies on the other hand a delay in the recording of the out flow corresponding to the time necessary for the ions to diffuse from the glandular opening to the gap The probe used is designed to give an accurate quantitative recording of the conductance change but other probe types which are better suited to reproduce the time course of the outflow can also be developed

The error involved in relating the conductance change exclusively to Na^+ and Cl^- ions has been discussed above Only by further chemical analyses preferably using radioisotope technique would it be possible to find out whether nerve stimulation may cause a minor increase in the outflow of other ions as well To determine if this is the case is however beyond the scope of this investigation

The chemical analyses have their sources of error as well *e g* the great risk of contamination when handling samples of low ionic concentration It is therefore not possible by comparing the results of chemical determinations and conductance measurements to draw any safe conclusion about the accuracy of the latter method

One source of error when determining the electrolyte concentration by means of its electrical conductivity is the influence of temperature changes the temperature coefficient of the conductivity being about 2 % per C Although care was taken to control temperature variations an error of a few per cent might still easily occur However to attain the very highest degree of accuracy has not been the main aim of the application of the conductance method For our present purposes an approximative determination within ± 10 % would be quite satisfactory Although we do not have enough material for a statistical proof we would estimate the methodological errors to be within this order of size

As already pointed out the conductance method has the advantage over the chemical analysis that it provides information about the total ionic concentration at the moment

when stimulation sets in This implies also that the conductance method permits determinations of the increases in ionic outflow during several consecutive stimulation periods without changing the compartment fluid as long as its concentration remains within the sensitivity range of the reflectometer

It is our experience from the series of experiments performed so far that the conductance measurements by means of high frequency reflectometry provides a simple index on glandular secretion which may be of use in many types of studies of frog skin gland secretion and might be of special value in comparative studies of electrical events associated with the secretion process

This work was supported by the Swedish Medical Research Council (project No 4490)

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Acta physiol scand 1975 93 77–84
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Comparative Studies on the Effects of Bradykinin and Vagal Stimulation on Motility in the Stomach and Colon

By

S Fasth L Hultén T Jahnberg and J Martinson

Received 31 May 1974

Abstract

Fasth S L Hultén T Jahnberg and J Martinson *Comparative studies on the effects of bradykinin and vagal stimulation on motility in the stomach and colon* Acta physiol scand 1975 93 77–84

The effect of bradykinin on gastric and colon c motility was studied in anaesthetized cats with volume recording devices and compared with the effects of vagal nerve st mulation When administered intra arterially bradykinin caused a profound and prolonged gastric relaxation Simultaneously there was a marked and likewise prolonged colonic contraction The gastric relaxation closely mimicked the atropine resistant relaxation el cited by vagal nerve stimulation These effects could not be blocked by antiadrenergic drugs and it is suggested that bradykinin and the unknown transmittor substance(s) released on vagal stimulation act in a similar way on the gastric smooth muscles and that a kinin mechanism may be involved in the vagal response As regards the colonic motor response it was shown that bradykinin does not re produce the vagal motility effects on colon smooth muscle but mimicks closely the atropine resistant e pulsive contraction el cited by activation of the pelvic nerves

Studies on the extrinsic nervous control of the motility in the stomach and colon indicate that specific parasympathetic fibre sets are involved in the receptive relaxation of the stomach and the expulsive colonic contraction (Martinson 1965 Hulten 1969) These motility re sponses elicited after vagal and pelvic nerve stimulation respectively appear to be neither pure cholinergic nor adrenergic After cessation of the stimulation the receptive relaxation and the colonic contraction are extremely slowly eliminated in contrast to most other types of nervous effector responses The transmittor mechanisms involved in these longlasting motor responses are obscure

The gastric receptive relaxation and the colonic expulsive contraction represents a spe cialized reservoir function in both organs In the stomach and the colon the respective responses are closely linked to a regional blood flow increase occurring concomitantly The transmission mechanism responsible for this functional hyperemia is also largely un known A plasma kinin has turned out to be involved in the atropine resistant functional vasodilatation of the cat s submandibular gland (Hilton and Lewis 1955 1956 Gautvik 1970 Gautvik Nysi

nism might be involved in functional hyperemia in the glandular tissue of the stomach and colon It has in fact recently been shown that bradykinin (Bk) can reproduce in detail not only the effect of pelvic nerve stimulation on the consecutive vascular sections of the colon but also the motor contraction which is closely linked to the vascular response (Fasth and Hultén 1973 a b)

The present study was undertaken to see whether Bk could also reproduce the receptive gastric relaxation occurring after efferent vagal stimulation

Material and Methods

Experiments were performed on 18 cats weighing 2 4–5 3 kg and fasted for 24 h The animals were anaesthetized intravenously with chloralose (50 mg/kg b w) after induction with ether A tracheal cannula was inserted to allow a free airway The right femoral artery was connected to a mercury manometer for recording of arterial pressure

Reco ding of gastric and colon c volume Since abdominal surgery interferes considerably with the gastric responses on vagal stimulation (Jansson 1969) another approach was preferred The gastric motility was recorded by means of a large thinwalled rubber balloon introduced in the stomach via esophagus The balloon was connected to a water filled reservoir of wide dimensions coupled to a piston recorder or to a force transducer (Grass force displacement transducer FT 10) operating a potentiometer writer (Servogor 2 C) By means of such an arrangement intragastric pressure could be kept constant at about 4 5 cm H_2O In 7 expts colonic motility was simultaneously recorded by means of a similar volume recording device A plastic tube was introduced into the distal colon via anus and connected to a waterfilled pressure reservoir The intraluminal pressure was kept constant at about 10 cm H_2O The pressure reservoir was coupled to a piston recorder operating on the kymograph (Fig 1)

Administ ation of d ugs To allow for close intraarterial BK administration a thin polyethylene catheter was inserted into the left femoral artery and passed upwards the aorta to a level some 3–4 cm above the celiac artery Bk (kindly supported by Bofors Nobel Pharma) was dissolved in 0 9 saline in concentra between 1 0–100 µg/ml and administered as bolus injections via the indwelling catheter In some expts Bk was also administered as i v injections Other drugs used were Atropine sulphate (Merck) guanethidine (Ismelin® Ciba) hexamethoniumchloride (Merck) phenoxybenzamine hydrochloride (Dibenzyline® Smith Kline & French) propranolol chloride (Inderal® Scanmeda) and histamine-chloride (ACO) In some experiments gallamine triethiodide (Flaxedil® May and Baker) was used to eliminate somatomotor reflex activity Artificial respiration was then maintained by a respiration pump

Ne rou stimulation The vagal nerves were in all experiments dissected free at the cervical level for subsequent stimulation The peripheral cut ends were then mounted on silver ring electrodes Stimulation parameters were usually 5 imp/s 5 ms 8 V delivered from a Grass stimulator model 5 SE

Results

1 The effect of intraaortic Bk injections and vagal stimulation on gastric and colonic volume

As is shown in Fig 2 intraaortic injection of Bk (10 µg) gave rise to a characteristic increase of gastric volume occurring within 5–10 s A slight relaxation was sometimes observed after 1 µg Bk In a dose of 100 µg Bk caused a somewhat more powerful response than did 10 µg Bk The higher dose was followed by a profound decrease in systemic arterial blood pressure however and was therefore avoided The relaxatory response always occurred rapidly and reached its maximum within about 10–20 s The gastric volume then decreased slowly and did not reach control level until 15–20 min had elapsed

Stimulation of the vagal nerves at high rates (usually 5 Hz 5 ms 8V) caused sometimes

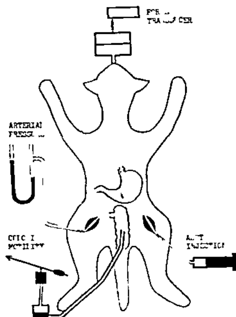


Fig 1 Schematic illustrat on of the techn que used for studying gastric and colonic volume changes

a slight immediate gastric contraction After cessation of the stimulation there was a powerful relaxation which in all respects closely mimicked the response observed on Bk injection (Fig 2) Quantitatively the change in gastric volume varied between 20–70 ml in different cats but was in a given animal always of the same magnitude as that induced by subsequent vagal nerve stimulation The relaxatory response was not abolished after Flaxedil[8] If any



Fig. 2. Cat 3 1 kg. The effect of intraaortic adm nistration of bradykinin (left panel) and vagal stimulation on gastric volume (right panel) Note the identical responses which are characterized by a rap d powerful relaxation and an extremely slow elimination

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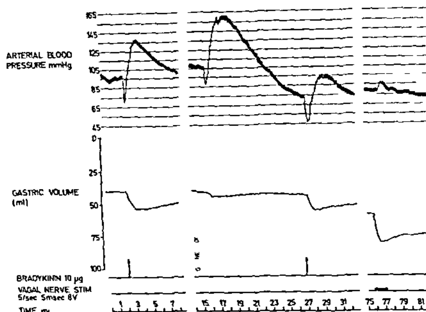


Fig 4 Cat 3 kg. The effect of bradykinin and vagal stimulation on gastric volume after guanethidine (5 mg/kg i v) The gastric relaxation elicited by bradykinin (middle panel) and vagal stimulation (right panel) are both unaffected

amounting to at the most 5 ml as compared with 70 ml on intraaortic administration developed more slowly and ceased within 3–4 min Furthermore it could be abolished after pretreatment with alfa and betaadrenergic blocking drugs Concomitant with the gastric relaxation there was an inhibition of the colonic motility

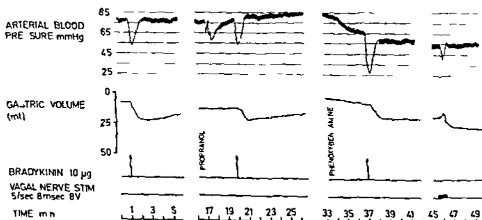


Fig. 5 Cat 8 kg The effect of bradykinin and vagal stimulation on gastric volume after propranolol (3 mg kg i v) and phenoxybenzamine (5 mg kg i v) Note that the relaxatory effects are still elicited after propranolol (middle panel) and phenoxybenzamine (right panel)

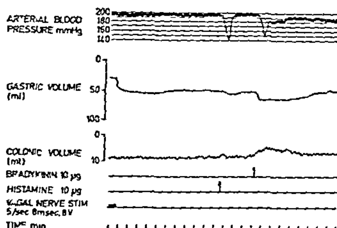


Fig 6 Cat 5 3 kg, atropinized (1 mg kg i v) The effect of intra aortic injection of histamine and bradykinin on gastric and colonic volume Note the insignificant motor effects after histamine compared to the protracted gastric relaxation and colonic contraction after bradykinin

III The effects of bradykinin and histamine on the motility in the stomach and colon For comparison the effects of intraaortic histamine injections were studied in 5 expts Histamine (10 µg) elicited a very moderate gastric relaxation and colonic contraction appearing within some 5-10 s As is shown in Fig 6 the responses differed also in many other respects from that following Bk Thus the gastric relaxation after histamine developed more slowly and returned to control level within 2-3 min The shortlasting decrease in colonic volume also differed considerably from that induced by Bk The gastric response to histamine was on the whole similar to that occurring after intravenously administered Bk

Discussion

In the present study it has been shown that Bk causes a gastric relaxation and colonic expulsive contraction which are qualitatively and quantitatively almost identical to the motor effects elicited by vagal and pelvic nerve stimulation respectively These nervous responses are considered to be involved in the gastric receptive relaxation and colonic expulsive contraction respectively (Martinson 1965 Hulten 1969)

Intraaortic Bk injections might hypothetically bring about gastric relaxation in different ways In most intestinal *in vitro* preparations Bk causes contraction sometimes preceded by shortlasting relaxation (Rocha e Silva Beraldo and Rosenfeld 1949 Konzett and Sturmer 1960 Turker Kiran and Kaymakcalan 1964 Fishloch 1966) In these studies Bk is supposed to exert its effects by a direct action on the smooth muscles. This is also in accordance with results presented by Gershon (1967) Bk has also been shown to inhibit even other smooth muscle preparations among other strips from the rat gastric fundus (Rocha e Silva 1953) Such a sympathicomimetic effect is neither caused by a local catecholamine release nor by interaction with the adrenergic receptors but may rather be dependent on the actual tone of smooth muscles (Antonia 1968 Hall and Bonta 1972) Studies *in vivo* suggest that Bk can stimulate nociceptive paravascular nerve endings evoking reflex inhibition either relaying at supraspinal or at ganglionic level with subsequent release into the blood stream of catecholamines from the adrenal medulla (Della Bella Benelli and Paoli 1972) It has also

been shown that Bk can inhibit smooth muscles by a direct effect on the ganglionic cells (Lewis and Reit 1965) Furthermore Bk has turned out to be a potent releaser of catecholamines by a direct action on the adrenal medulla (Feldberg and Lewis 1964) In fact it has recently been shown that Bk when administered intravenously causes a profound intestinal inhibition which is abolished after adrenalectomy (Fasth and Hulten 1973 a)

In the present study the gastric relaxation on intraaortic Bk injection which occurred within some 5–10 s was not abolished by antiadrenergic drugs and differed largely from that evoked by adrenaline and noradrenaline (Martinson 1965) It therefore appears unlikely that the response is caused by an indirect reflex release of catecholamines from the adrenal medulla As compared with the effects of intraaortal administration the moderate gastric relaxatory effect of Bk injected intravenously occurred after a long latency and the colonic motility recorded simultaneously was then slightly inhibited It therefore appears likely that this response was caused by an adrenergic mechanism This is in accordance with a previous study (Fasth and Hultén 1973 a) which showed evidence that Bk administered intravenously inhibits intestinal motility while intraarterial administration regularly gives rise to increased motility

The gastric relaxation and colonic contraction elicited by Bk could not be blocked by hexamethonium indicating that Bk does not exert its effects by a direct action on the ganglionic cells In this context it should be mentioned that the contraction of the nictitating membrane in cats in response to Bk though unaffected by hexamethonium was nevertheless shown to be caused by a direct stimulation of the superior cervical ganglion (Lewis and Reit 1965) The concentration of Bk exceeded considerably that in the present study and the motor response differed largely both qualitatively and quantitatively The results of the present investigation therefore indicate that the gastric relaxation elicited by intraaortic Bk injection is neither caused by an adrenergic mechanism nor by a direct ganglion stimulating effect but is rather due to a direct effect on the gastric smooth muscles

The transmittor substance released and responsible for the potent motor effects elicited via vagal and pelvic nerves is as mentioned still unknown In view of the present results it may be tempting to believe that a kinin like substance is involved Since the motor responses are characteristically protracted local release of such specific transmittor agents must obviously be eliminated and/or inactivated at a very slow rate Kinins are rapidly inactivated in the organism by peptidases however (Erdos 1966) It therefore appearsless likely that the longlasting motor responses observed in the stomach and colon are due to continous action of the peptide per se unless some unknown mechanism interferes with its inactivation or elimination Another explanation might be that these prolonged motor effects are induced along other pathways outside or within the smooth muscle cells As regards the stomach it has been suggested that intramural nonadrenergic inhibitory neurons or purinergic nerves are involved in the vagal gastric relaxation (Burnstock 1972) As regards the longlasting colonic contraction such a mechanism is less likely however since the purinergic nerves exert a similar relaxatory effect on the colonic smooth muscles and lack extrinsic nervous influence

It has previously been shown that Bk can in detail reproduce the effect of pelvic nerve stimulation on colonic motility and blood flow (Fasth and Hultén 1973 a b) and even the

effect of vagal stimulation on gastric circulation at least partly (Fasth and Martinson 1973) In the present series of experiments Bk was also shown to reproduce the vagally induced gastric relaxation Further investigations are necessary to elucidate whether a kinin like substance is in fact involved in the nervous regulation of the stomach and colon

This research has been sponsored from the Swedish Research Council (B72 17X 3176-07) from the Faculty of Medicine University of Goteborg and from Goteborgs Läkaresällskap

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Acta physiol scand 1975 93 85–96

From the Department of Physiology University of Göteborg, Sweden

Aspects of the Central Integration of Arterial Baroreceptor and Cardiac Ventricular Receptor Reflexes in the Cat

By

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Received 4 June 1974

Abstract

LITTLE R G WENNERGREN and B ÖBERG *Aspects of the central integration of arterial baroreceptor and cardiac ventricular receptor reflexes in the cat* Acta physiol scand 1975 *93* 85–96

The possible central integrative mechanisms responsible for the earlier reported differentiated reflex engagement of the renal and muscle vessels and the heart from cardiac ventricular receptors and arterial baroreceptors respectively were analyzed in atropinized cats The reflex renal vessel muscle vessel and heart rate responses expressed as per cent of maximum to graded activations of arterial baroreceptors (sinus pressure variations) and stimulations of ventricular receptor afferents in the cardiac nerve were systematically compared Cardiac nerve stimulation with low frequencies was found to elicit more pronounced reflex renal vessel responses than muscle vessel and heart rate responses In contrast elevations of sinus pressure induced equally pronounced renal and muscle vessel responses High frequency cardiac nerve stimulation elicited maximal reflex renal vessel responses but only submaximal effects on muscle vessels and heart rate while intense baroreceptor stimulation induced maximal reflex effector responses throughout The submaximal heart rate response to cardiac nerve stimulation is probably due to a simultaneous activation of excitatory afferents On the other hand the less pronounced muscle than renal vessel responses when the cardiac nerve was stimulated probably reflect a relatively sparse innervation of muscle vasomotor neurons by ventricular receptor afferents which seem instead to be preferentially oriented towards renal vasomotor and possibly cardiac motor neurons

Recent studies have shown that stimulation of left ventricular distension receptors (Öberg and Thorén 1973 a) or direct electrical stimulation of their non medullated afferents (Öberg and White 1970 Öberg and Thorén 1973 b) induces marked cardiovascular depressor responses qualitatively similar to those induced from the arterial baroreceptors When however the reflex effects originating from these two groups of receptors were compared in more detail clearcut quantitative differences in the involvement of various cardiovascular target organs were seen It was noted that for a given reflex vasodilator response in the skeletal muscles the simultaneously evoked vasodilatation in the kidney was significantly

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more pronounced when the ventricular receptors were activated than when the baroreceptors were stimulated. It was also noted that the vagal control of the heart rate was more powerfully influenced from the ventricular receptors than from the arterial baroreceptors.

The main purpose of the present series of experiments was to analyze the possible background for this non uniform reflex engagement of the renal and muscle vascular beds and also to estimate whether the ventricular receptors had a particularly strong influence on the sympathetic control of the heart as well. To that end the reflex renal and muscle vasodilator responses and changes in heart rate after atropinization of the animal were followed when well-defined graded stimulations were applied to the carotid sinus baroreceptors and the non medullated afferent fibres from the heart.

Methods

Experiments were performed on 14 cats anesthetized with chloralose (30–50 mg/kg b.wt.). A tracheal cannula was inserted and the carotid arteries and the vago-sympathetic trunks on both sides of the neck were exposed. The aortic nerves were identified and cut at their junction with the superior laryngeal nerves; the latter nerves were also sectioned bilaterally. The left vagal nerve was cut in the neck. The animals were placed on positive pressure artificial respiration and ventilated with 97 O_2 and 3 CO in most experiments. The chest was opened by means of a right intercostal incision in the 5th interspace. The azygous vein was divided and the right cardiac nerve, running from the heart to the vagal stem in the chest, was freed, cut distally and placed on an electrode for electrical stimulation. The carotid sinus regions on both sides were isolated by ligation of the main arterial branches in these areas although, to avoid damage to the sinus nerves, no attempt was made to isolate the sinuses completely by ligation of the minor vessels accompanying these nerves.

The carotid sinus regions were perfused with blood via a Harvard peristaltic pump (Model 500 1200). Blood was usually taken from the superior mesenteric artery, which was cannulated after opening the abdomen and removal of the intestine, the omentum and the spleen. From the pump, the blood perfused the sinus regions through twin catheters placed in a cephalad direction in the common carotid arteries just below the sinuses. A Windkessel reservoir was connected to the system via a rubber T tube so that the sinus regions could be exposed to either pulsatile or nonpulsatile pressures. The blood from the sinus regions was returned to the animal via catheters from the external carotid arteries to the right external jugular vein. The outflow resistance from the sinus regions, and thereby the intrasinusal pressure, could be varied over a wide range by adjustment of a screw clamp on the external carotid artery-jugular vein shunt. The intrasinusal pressure was recorded with a Statham transducer from a side branch on the outflow tubing close to the sinus regions.

Systemic blood pressure was recorded from one femoral or brachial artery with a Statham transducer on a Grass Model 5 Polygraph recorder. Heart rate was monitored on the polygraph by a tachograph, triggered by the rapid systolic rise of the arterial blood pressure. Blood flow from the left kidney was measured by cannulating the renal vein and passing the outflow through a drop recorder operating an ordinate writer on the Polygraph. The blood was returned to the animal through the superior mesenteric vein. Calf muscle blood flow was similarly measured as the venous outflow from the popliteal vein. The paw was excluded from the circulation by mass ligatures. The arterial blood pressure could be maintained constant during the experiment by connecting the arterial side of the circulation via one femoral artery to a pressure compensator.

Heparin (5 mg/kg b.wt.) was used as an anticoagulant. Atropine (0.3–0.5 mg/kg b.wt.) was given to all animals.

Experimental procedure and treatment of the data. The speed of the pump perfusing the sinus regions was initially adjusted so that with complete occlusion of the outflow tubing, a high intrasinusal pressure (usually 50–75 mm Hg) was obtained. By adjustments of the screw clamp, the intrasinusal pressure could then be varied in steps from low values (5–10 mm Hg) up to this high level. The reflex vascular responses were measured in terms of changes in flow conductance (flow (ml/min)/pressure (mm Hg)). The values for heart rate and conductance at low sinus pressures (below 5 mm Hg) were taken as control

Responses obtained at very high pulsating sinus pressures and which did not increase on further elevations of the sinus pressure were set as maximum responses (100%). The responses at intermediate pressures were expressed as per cent of maximum values. — With the intrasinusal pressure kept low and thus with a minimum of baroreceptor activity the right cardiac nerve was then stimulated with intensities known to excite essentially all non medullated afferents (Öberg and Thorén 1973 b) usually 4 V and 2 msM duration and with stimulation rates increasing in steps from 1 Hz to 16 Hz. Again the heart rate and conductance values at 0 Hz was taken as control and the maximum responses obtained as the 100% response. — For statistical analysis mean values and S.E. of the mean were calculated and t test applied when comparing group of data.

Results

Effector responses to arterial baroreceptor stimulation

Fig. 1 A shows the relationships between sinus pressure and the reflex responses of the muscle and the renal vascular beds and heart rate. The data are based on repeated tests in 14 animals. It is seen that the sinus pressure reflex response curves for the renal and the muscle vascular beds have an identical course over the whole sinus pressure range. In other words activation of the carotid sinus baroreceptors produces equally pronounced reflex responses in the two vascular beds when expressed as per cent of the maximal response which could be induced reflexly from the baroreceptors. The heart rate response curve obtained in these atropinized animals is however significantly displaced to the right at sinus pressures below 200 mm Hg. Thus submaximal stimulation of the baroreceptors produces less intense reflex heart rate responses than muscle and renal vessel responses. These results confirm earlier observations by Kendrick *et al.* (1972).

Effector responses to cardiac nerve stimulation

Stimulation of the right cardiac nerve with intensities that activate the non medullated fibre group (Öberg and Thoren 1973 b) elicited reflex vasodilator responses in the kidney and the calf skeletal muscle and a moderate reflex slowing of the heart in the atropinized animals. The responses increased with increasing stimulation frequencies reaching a maximum at 10–12 Hz and then declined when stimulation frequency was further increased (Fig. 1 B and 1 C).

Comparisons of the curves relating reflex effector response as per cent of maximum to the frequency of afferent stimulation revealed that the frequency reflex response curves for the muscle vessels (Fig. 1 B) and heart rate (Fig. 1 C) were significantly displaced to the right of that for the renal vessels in the low stimulation frequency range (*i.e.* below 4 and 6 Hz, respectively). Within these low frequency ranges the reflex renal responses expressed as per cent of maximum were thus significantly greater than the muscle vessel and heart rate responses.

Comparisons of the effector responses to baroreceptor and cardiac nerve stimulations

When the *maximal* reflex responses obtained with cardiac nerve stimulation on one hand and with baroreceptor stimulation on the other were compared in one and the same animal it was found that the maximal *renal* responses were similar. The maximal reflex increase of renal conductance induced by cardiac nerve stimulation thus averaged 88 % ±

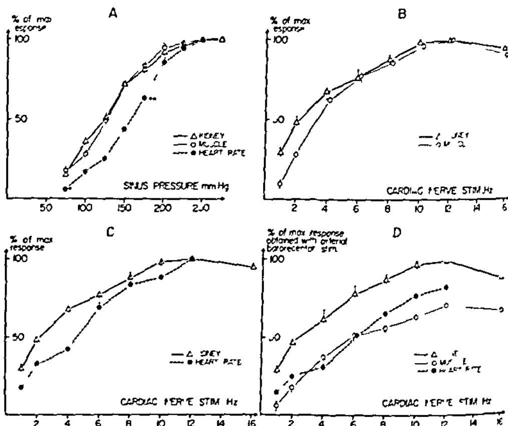


Fig. 1 (A) The relation between the pressure level in isolated carotid sinus regions and muscle vessel, renal vessel and heart rate responses expressed as per cent of maximum The muscle and renal vascular responses agree over the whole range of sinus pressures while the heart rate data (statistically compared with the muscle vessel data) are significantly displaced below and to the right

(B) Relation between rate of stimulation of the cardiac nerve and muscle and renal vessel responses in per cent of maximum The muscle vessel response curve is significantly displaced to the right of the renal vessel curve in the low frequency range (4 Hz)

(C) Relation between rate of stimulation of the cardiac nerve and renal vessel and heart rate responses. The heart rate response curve is significantly displaced to the right in the low frequency range (6 Hz)

(D) Relation between rate of stimulation of the cardiac nerve and effector responses expressed as per cent of the maximal response that could ever be reflexly induced i.e. with intense baroreceptor stimulation The muscle vessel response curve is significantly displaced to the right of the corresponding curve for the renal vessels over the whole range of stimulation frequencies Also the heart rate response curve is displaced to the right as was the case with baroreceptor stimulation (Fig 1 A) The data represent mean values from multiple tests in 14 animals vertical bars ± S.E. p 0 05 p 0 01 p 0 001

10 % (S E.) in the 14 animals while the maximally obtained response to arterial baroreceptor stimulation averaged 89 14 %. In contrast the maximal reflex increase of muscle vessel conductance obtained when the cardiac nerve was stimulated was significantly smaller than that resulting from arterial baroreceptor stimulation averaging 30 % 77 and 465 112 % respectively (p 0 01)—A record demonstrating this is shown in Fig 2 In this experiment maximal renal and muscle vessel responses to cardiac nerve stimulation were first produced after which a powerful baroreceptor stimulation was superimposed by elevating the intrasinusal pressure to high levels It is seen that this latter manoeuvre induced a

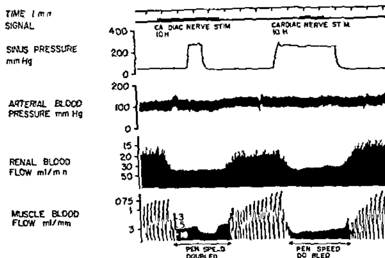


Fig 2 Renal and muscle vessel responses when an intense baroreceptor stimulation is superimposed on a *maximal cardiac nerve stimulation (left) and vice versa (right)* Note that baroreceptor stimulation produces maximal responses in both vascular beds (no further effect of the superimposed cardiac nerve stimulation) while only submaximal muscle vessel responses are obtained with cardiac nerve stimulation (a clear cut additional dilatation is produced by the superimposed baroreceptor stimulation)

clearcut further dilatation in the skeletal muscle while no additional reflex dilator response was observed in the kidney When a maximal cardiac nerve stimulation was added to a maximal baroreceptor activation no reflex effects of the cardiac nerve stimulation were seen

A similar discrepancy was also found with regard to the maximal heart rate responses which could be reflexly induced by cardiac nerve stimulation and by baroreceptor stimulation The maximal reduction in heart rate with arterial baroreceptor stimulation averaged 29 1 ± 4 2 beats/min (or − 12 6% ± 1 4 %) while the corresponding figures with cardiac nerve stimulation were 21 ± 2 7 beats/min (or − 9 3 % ± 1 3 %) ($p < 0.05$) The modest heart rate responses to cardiac nerve stimulation in the present study should be compared with the very marked reflex effects obtained in animals with intact efferent vagal innervation to the heart where a reduction of heart rate of 100 beats/min or more was usually obtained as a maximum response (Öberg and Thoren 1973 b)

If the reflex effector responses are again related to the rate of afferent stimulation of the cardiac nerve but now taking into account the above mentioned observations that such stimulations are capable of producing only *submaximal* muscle vessel and heart rate responses the frequency response curves shown in Fig 1 D are obtained In this figure the effector responses to cardiac nerve stimulation are thus expressed as per cent of the *maximal reflex response* which could ever be obtained *i e* the response to intense baroreceptor stimulation — The muscle vessel response curve is then seen to be clearly displaced to the right of the renal vessel curve over the whole frequency range this in contrast to the close agreement between the sinus pressure reflex response curves for the two vascular beds (Fig 1 A) The heart rate response curves on the other hand are in both cases displaced in a similar fashion to the right of the renal vessel response curves (*cf* Fig 1 A and 1 D)

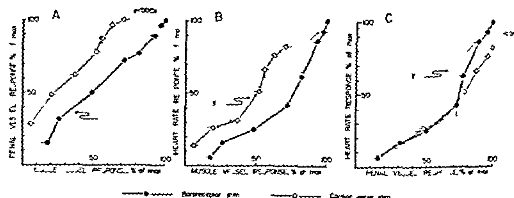


Fig. 3 Relation between reflex muscle vessel and renal vessel responses (A) reflex muscle vessel and heart rate responses (B) and reflex renal vessel and heart rate responses (C) when the cardiac nerve (open symbols, broken line) and the arterial baroreceptors (filled symbols, solid line) were stimulated The effector responses (mean ± S E.) are expressed as per cent of the maximal reflex response which could ever be induced in each individual experiment *i.e.* during intense baroreceptor stimulation Note that for a given muscle vessel response the renal vessel and heart rate responses are more pronounced when the cardiac nerve is stimulated than when the baroreceptors are activated

The data shown in Figs 1 A and 1 D have been combined in constructing Fig 3 which describes the relationships between the reflex renal and muscle vessel responses (A) the reflex heart rate and muscle vessel responses (B) and the reflex heart rate and renal vessel responses (C) following stimulation of the cardiac nerve (open symbols broken lines) and of the arterial baroreceptors (filled symbols solid lines) The effector responses are expressed as per cent of the maximal reflex responses obtained with intense baroreceptor stimulation Fig. 3 A demonstrates that while the reflex renal and muscle vessel responses are closely similar when the baroreceptors are stimulated (*cf* Fig 1 A) and the symbols therefore fall practically on the identity line the corresponding data when the cardiac nerve was stimulated are significantly displaced upwards and towards the left reflecting the more pronounced renal vessel engagement in this situation The diagram of Fig. 3 B where the muscle vessel and heart rate responses have been related in a similar way shows that with cardiac nerve stimulation the data are displaced above and to the left of the baroreceptor data just as was the case when the reflex renal and muscle vessel responses were compared in panel A This indicates that when the two receptor groups are activated to induce reflex muscle vessel responses of similar size the sympathetic cardio-accelerator fibre activity like the renal vasoconstrictor fibre activity is more powerfully influenced from the ventricular receptors than from the arterial baroreceptors However as mentioned above, cardiac nerve stimulation doe not induce maximal reflex responses eith r in skeletal muscles, or with respect to heart rate

When finally the reflex heart rate and renal vessel responses are similarly related to each other in the two reflex mechanisms (panel 3 C) it is seen that the curves have an essentially identical course at least as long as the reflex effector responses are not close to maximum. This indicates that, for a given effect on the renal vasomotor neurons the cardio-accelerator fibres are engaged to the same extent in the two reflex mechanisms When however the

reflex effector responses approach maximum levels the heart rate response becomes relatively less pronounced upon cardiac nerve stimulation than when the baroreceptors are activated

Discussion

The present series of experiments were undertaken in an attempt to analyze more closely the possible background for the earlier reported observation that the renal vessels appeared to be more powerfully engaged in reflexes emanating from cardiac ventricular receptors than in arterial baroreceptor reflexes (*e g* Öberg and Thorén 1973) This latter conclusion was based on results from experiments where the size of the reflex renal responses were compared when the two sets of receptors were stimulated to induce equally large reflex responses in the skeletal muscle vascular bed

The present results indicate that the particularly strong renal vessel engagement in ventricular receptor reflexes depends on two fundamental mechanisms *Firstly* the curve relating the magnitude of the reflex renal vasodilator response to frequency of cardiac nerve stimulation was found to be steeper than the corresponding curve for muscle vessels in the lower range of afferent fibre discharge (below 4 Hz) Thus within this low frequency range the reflex renal vessel responses will be larger than muscle vessel responses when expressed as per cent of maximal response This is in contrast to the situation when the arterial baroreceptors are activated in which case the stimulus reflex response curves for the two vascular beds have an identical course and the muscle and renal vessel responses to a given change in the sinus pressure therefore are similar throughout

Secondly while an intense activation of the cardiac receptors (or their afferents) seems to cause a complete cessation of activity in the renal vasomotor neurons such an afferent stimulation is evidently not capable of inducing a total reflex inhibition of the vasoconstrictor fibre activity to the skeletal muscles In contrast a powerful excitation of the arterial baroreceptors completely inhibits the impulse activity in both sets of vaso constrictor fibres

These mentioned differences in ventricular receptor and arterial baroreceptor influences on renal and muscle vasomotor neurons result in the relationships illustrated in Fig 3 Panel A of this figure demonstrates that when the cardiac nerves (or the ventricular receptors) are stimulated to induce a reflex skeletal muscle vessel response amounting to 20–25% of maximum there is a concomitant renal vessel response which corresponds to approximately 50 % of maximum When on the other hand the arterial baroreceptors are stimulated to produce a similar reflex muscle vessel effect the accompanying reflex renal vessel response now amounts to only 20–25 of maximum The data shown in the diagram in Fig 3 A which is constructed from the stimulus reflex response curves obtained when the baroreceptors and the cardiac nerves were stimulated in 14 animals are thus in complete agreement with the earlier observations that for a given reflex muscle vessel response the reflex renal responses are more pronounced when the cardiac nerve (or ventricular receptors) are stimulated than when the baroreceptors are activated (*e g* Öberg and White 1970 Öberg and Thorén 1973 a)

The relatively strong reflex influence from the ventricular receptors on renal vasomoto

activity compared with that in skeletal muscle is thus according to the present experiments, only in part due to a more intense engagement of the renal vasomotor fibres *per se* The main reason for the differentiated engagement of the two vascular beds in the baroreceptor and the cardiac receptor reflexes is in fact that the *muscle vasomotor fibres* are *less* engaged than the renal vasomotor nerves in ventricular receptor reflexes a phenomenon which becomes apparent first when the two reflex mechanisms are maximally activated Thus to be precise only when cardiac receptor activity varies within the narrow range of low discharge rates where the reflex response curve for the renal vessels is considerably steeper than the muscle vessel curve (Fig 1 B) one is justified to ascribe the differentiated reflex engagement of the two vascular beds to a more pronounced renal vessel response However this low frequency range is probably the physiologically relevant one since recordings of afferent impulse activity from the ventricular receptors have shown that they rarely fire at rates higher than 1–2 Hz in control situations in fact many receptors are then completely silent (Öberg and Thoren 1972) Even if these measurements were made on open chest animals probably displaying an artificially low receptor activity due to a shrinkage of the heart (Rushmer Finlayson and Nash 1954) it is very likely that the ventricular receptor firing rate normally varies essentially within the very range where the renal vessel response curve is particularly steep

Earlier studies have also shown that when the arterial baroreceptors and the ventricular receptors (or their afferents) are stimulated to produce similar reductions in blood pressure or muscle flow resistance the accompanying reflex slowing of the heart in the non atropinized animal is significantly more pronounced when the ventricular receptors were activated (Öberg and White 1970 Öberg and Thorén 1973 a) This discrepancy with regard to the heart rate responses in the two reflex mechanisms has been ascribed to a particularly strong reflex engagement of the vagal fibres to the heart from the ventricular receptors The results of similar comparisons on atropinized cats in the present study (Fig 3 B) suggest that the latter receptors also influence the sympathetic cardio-accelerator outflow reflexly more than the arterial baroreceptors It is thus clear from the displacement of the baroreceptor and ventricular receptor curves in Fig 3 A and 3 B that a differentiated engagement of the *renal and muscle vasoconstrictor fibres (3 A) and of the sympathetic cardio-accelerator* and muscl vasoconstrictor fibres (3 B) occur in baroreceptor and ventricular receptor reflex patterns In contrast the sympathetic efferent fibres to the heart and the renal vessels seem to be mutually engaged to the same extent in both ventricular receptor and baroreceptor reflexes (Fig 3 C) provided the effector responses are not close to maximum In this latter case the cardio-accelerator fibre activity seems to be relatively less influenced from the cardiac receptors A possible explanation for this phenomenon will be discussed below

The displacement of the reflex heart rate response curve to the right in both baroreceptor and ventricular receptor reflexes (Fig 1 A and 1 C) is as has been discussed in an earlier publication (Kendrick *et al* 1972) probably due to the fact that maximal heart rate responses are already obtained at low discharge rates in the cardio-accelerator fibres Thus 80 90 of the maximal heart rate response is obtained when the efferent sympathetic fibres to the heart are stimulated at rates around 3–4 Hz (Folkow Lofving and Mellander 1956 Lindgren and Manning 1965) Only small changes in heart rate will therefore occur when

the impulse frequency is altered in the range above 4 Hz A relatively strong inhibitory influence from the two sets of receptors i e a relatively high sinus pressure and high frequency of stimulation of the cardiac nerve causing the impulse rate in the cardio-accelerator fibres to decrease below 3–4 Hz, is evidently required before clearcut heart rate changes can be expected to occur In contrast the muscle and renal vascular beds show maximal responses at considerably higher discharge rates around 12–16 Hz (*cf* Celander 1954 Mellander 1960) and distinct reflex vascular responses should therefore occur even with a moderate inhibition of vasoconstrictor fibre activity i e with low intrasinusal pressures and low rates of cardiac nerve stimulation

There is at present very limited information on how the interneuronal connections between the afferent fibres and the motor neurons in the vasomotor centre are functionally organized to account for the differentiated engagement of various cardiovascular target organs in ventricular receptor and baroreceptor reflex mechanisms Both baroreceptor reflexes and reflexes from cardiac receptors seem to be mediated through the same structures in the medulla oblongata (Lee Kuo and Chai 1972) i e the nucleus tractus solitarius (Humphrey 1967 Seller and Illert 1969) Besides this general outline little is known concerning the projections of the two sets of afferents within the vasomotor centre and the detailed arrangement of their synaptic contacts with the various autonomic motor neurons in this structure The results of the present analysis suggest however that quantitative differences may exist with regard to the density of innervation of *e g* renal and muscle vasomotor neurons from the two respective afferent systems The steeper frequency response curve for the renal vessels when the cardiac nerve was stimulated with low frequencies (Fig 1 B) may thus indicate that the renal vasomotor neurons or at least a portion of them are more extensively supplied with cardiac receptor afferents than the muscle vasomotor neurons In fact the observation that maximal inhibition of muscle vasomotor fibre activity could not be produced even by intense cardiac nerve stimulation is compatible with the idea that some of the skeletal muscle vasomotor neurons lack innervation from cardiac receptor afferents Earlier findings also seem to indicate that differences exist with regard to the number of afferent fibres reaching the various motor neurons in the vasomotor centre from the two respective groups of receptors The reflex bradycardia induced in the non atropinized cats by maximal baroreceptor stimulation is thus clearly submaximal when compared with the effects induced from the ventricular receptors (*e g* Öberg and White 1970 Öberg and Thorén 1973 a) suggesting that the cardiac vagal motor neurons are more abundantly supplied with ventricular receptor afferents than baroreceptor afferents Furthermore medullated and non medullated baroreceptor afferents evidently differ with respect to their orientation among the various neuron pools in the vasomotor centre the non medullated fibres being more abundantly distributed to the cardiac vagal moto neurons (Kendrick *et al* 1974)

When discussing the possible functional significance of the findings in the present study it should be pointed out that when the two reflex mechanisms were compared the reflex was in one case elicited by application of the natural stimulus to the endings but in the other case by electrical stimulation of the afferent pathways It would have been more satisfactory if in the present analysis also the ventricular receptor reflex had been

elicited by stimulating the endings adequately since the electrical stimulation of the cardiac nerve involves certain obvious drawbacks Thus several afferent systems both inhibitory and excitatory are simultaneously activated (Öberg and Thorén 1973 b) Furthermore all stimulated afferents will discharge at uniform rates while normally the afferent activity is in all likelihood augmented both by recruiting more receptors and by increasing the firing rate in each individual receptor afferent fibre unit However in view of the technical difficulties involved when a selective and well graded adequate stimulation of the ventricular receptors is desired it was considered justifiable to resort to the present simplified approach and particularly so since the reflex responses to ventricular receptor activation and to electrical stimulation of the cardiac nerve with suitable stimulation characteristics are qualitatively similar (Öberg and Thorén 1973 a and b) The decline of the reflex vascular responses which occurred in parallel in the kidney and the skeletal muscle when the cardiac nerve was stimulated with higher frequencies may however be a consequence of the artifical electrical stimulation and due to a growing impact from simultaneously activated excitatory medullated fibres when the stimulation frequency is increased above 10–12 Hz (Öberg and Thorén 1973 b) This excitatory influence seems to be rather modest as far as the effects on the vascular beds are concerned and of essentially the same order of magnitude for renal and muscle vessels (Öberg and Thorén 1973 b) The failure to induce a maximal reflex vasodilator response in the skeletal muscles when the cardiac nerve was stimulated while at the same time a maximal renal vasodilatation was present can therefore reasonably not be ascribed to the simultaneous activation of excitatory afferents in the cardiac nerve Finally even if it is possible that the abnormal and stereotype afferent discharge pattern produced by electrical stimulation of the cardiac nerve may to some extent influence the vasomotor centre response to the afferent signals it seems likely that *e g* the renal and muscle vasomotor neurons would then be influenced in a similar fashion The non uniform engagement of the two vascular beds in response to cardiac nerve stimulation can therefore in all probability not be explained by the artificial electrical stimulation *per se* but is rather due to differences with regard to the extent of afferent innervation of the two neuron pools as discussed above

On the other hand the clearly submaximal inhibition of sympathetic cardio-accelerator fibre activity when the cardiac nerve was stimulated to produce maximal renal vessel responses may very well be ascribed to the unavoidable simultaneous activation of excitatory afferents in the cardiac nerve and does not necessarily mean that the cardio-accelerator neurons are more sparsely supplied with afferents from the ventricular receptors than from the baroreceptors as is evidently the case with the muscle vessel vasomotor neurons The medullated excitatory fibres in the cardiac nerve which to a large extent probably emanate from atrial receptors (Öberg and Thorén 1973 b) thus seem to exert a particularly marked excitatory influence on sympathetic cardio-accelerator neurons (Ledsome and Linden 1964 1967 Öberg and Thorén 1973 b) and will therefore reasonably play a more decisive role in counteracting inhibitory influences on heart rate than on the muscle and renal vascular beds Since the excitatory effects of medullated fibre activation will assert itself first at stimulation rates around 8 10 Hz (Öberg and Thorén 1973 b) one would also expect that a clearcut interference with the inhibitory effects should first occur at high frequency

stimulation of the cardiac nerve That this is indeed the case is evident from the data in Fig 3 C demonstrating that when the cardiac nerve and the arterial baroreceptors are stimulated to produce identical renal vessel responses the concomitant reflex heart rate responses are also equally pronounced in the two reflex mechanisms as long as the reflex responses are not close to maximal *i e* when the cardiac nerve is stimulated with such low frequencies that the excitatory influence from the medullated fibres is negligible When however the reflex responses are close to maximum *i e* when the cardiac nerve is stimulated with higher frequencies the heart rate responses for a given reflex renal vessel response becomes decidedly smaller when the cardiac nerve is stimulated than when the arterial baroreceptors are activated In other words the heart rate responses to cardiac nerve stimulation become relatively smaller when the stimulation frequency is high enough to make the excitatory influence from the medullated afferents on sympathetic cardio-accelerator neurons effective The data in Fig 3 C are therefore compatible with the idea that the submaximal heart rate responses to high frequency stimulation of the cardiac nerve is due to opposing excitatory influences rather than to a less extensive innervation of the sympathetic neurons from the ventricular receptors than from the baroreceptors The fact that the curves in Fig 3 C have an almost identical course in the lower response ranges suggests that the sympathetic cardio accelerator neurons are indeed as extensively supplied with ventricular receptor afferents as the renal vasomotor neurons

From the findings in the present study it thus seems as if marked quantitative differences may exist with regard to the number of afferent fibres from the two sets of receptors impinging upon the different neuron pools in the medullary vasomotor centre According to this concept baroreceptor afferents taken as a group and including both medullated and non medullated fibres seem to be relatively homogeneously distributed among all neuron pools in the vasomotor centre and make contact with essentially all sympathetic vasomotor and cardio-accelerator neurons The efferent vagal fibres to the heart appear however to be less extensively innervated from baroreceptor afferents at least as far as the medullated fibres are concerned (Kendrick *et al* 1974) The ventricular receptor afferents on the other hand are evidently preferentially oriented towards renal vasomotor neurons and cardiac sympathetic and cardiac vagal motor neurons while the skeletal muscle vasoconstrictor fibres are more sparsely innervated from ventricular receptors In fact some skeletal muscle vasomotor neurons seem to completely lack synaptic connections with ventricular receptor afferents Whether this is the case also with the sympathetic vasoconstrictor fibres running to other circuits like the intestinal and the cutaneous vascular beds is not known

The preferential reflex engagement of the renal vascular bed from the ventricular receptors suggests that these endings are involved in a volume regulating cardio-renal reflex mechanism through which renal excretory function may be adjusted according to the prevailing diastolic filling and distension of the left ventricle (Öberg and Thoren 1973 a)

This study was supported by grants from the Swedish Medical Research Council (B74 14X-644 10C) from the Faculty of Medicine University of Goteborg from the Medical Research Council London from Magnus Bergvalls Stiftelse and from Wilhelm and Martina Lundgrens Vetenskapsfond

Thanks are due to Mrs Gertrud Karlsson and Mrs Eva Cari Einarsson for skilful technical assistance

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Measurement of Endometrial Blood Flow in Guinea-Pig Uterus by Clearance of Intraluminally Applied ¹³³Xenon Effects of Oophorectomy, Estrogen Treatment and Pregnancy

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Received 12 June 1974

Abstract

Einer Jensen N L Edvinsson B Falck Ch Owman N O Sjoberg and B Walles *Measurement of endometrial blood flow in guinea pig uterus by clearance of intraluminally applied ¹³³xenon effects of oophorectomy estrogen treatment and pregnancy* Acta physiol scand 1975 *93* 97–103

Endometrial blood flow (EBF) was studied atraumatically in guinea pigs by the ¹³³xenon clearance technique With the aid of an otoscope a xenon saline solution was deposited via the vagina through a cannula into the uterine lumen Radioactivity curves were obtained by a small external scintillation detector Oophorectomy lowered total flow whereas this was markedly increased (also per unit weight) by estrogen treatment, alone or in combination with oophorectomy During early pregnancy there was a tendency to increase in the flow while the values (for the parietal decidua) were markedly reduced at later stages of pregnancy In the fetus placenta unit (¹³³xenon solution injected intra amniotically) the flow was three times higher than in the parietal decidua at this advanced stage of pregnancy

Combined fluorescence histochemistry and chemical studies have shown that the mammalian uterus receives an ample supply of noradrenaline-containing nerves (see Sjoberg 1967 Marshall 1970 Owman Sjoberg and Sjostrand 1974) The majority of the nerves originate in ganglia at the utero-vaginal junction (Owman Rosengren and Sjoberg 1966 Owman and Sjoberg 1966) and constitute an anatomically and functionally unique system of "short adrenergic neurons innervating the myometrial smooth musculature (Owman *et al* 1966 1974) There is reason to believe that the remainder of the uterine adrenergic nerves which are vasomotor belongs to the classical sympathetic system and thus arise in pre and paravertebral sympathetic ganglia (Thorbert Alm Owman and Sjoberg, unpublished) Such vasomotor nerves occur mainly in the myometrial vascular bed but are also numerous in the endometrium (Falck *et al* 1974) The endometrial circulation is particularly well suited for quantitative measurements with the ¹³³xenon clearance technique after intraluminal application of the radioisotope (Secher Einer Jensen and Juhl 1973) and by this route traumatic interference with the local circulation is entirely avoided This

vasculature was chosen as model because it is influenced both by hormonal mechanisms a d by the vasomotor sympathetic nerves Oophorectomy estrogen treatment and pregnancy were used as experimental conditions to induce alterations in the blood flow

Material and Methods

40 adult nulliparous guinea pigs weighing approximately 350 g, were used in the flow measurements. They were maintained on a standard pellet diet and tap water *ad l b* 14 animals were oophorectomized by bilateral removal of the ovary through lumbar incisions under ether anesthesia 3 weeks prior to the endometrial blood flow (EBF) measurements During the last week 8 of these animals received a da? s c injection of 0.5 μg 17 β-estradiol benzoate Flow was also determined in 6 non-oophorectomized animals (in diestrus as checked by stained vaginal smears) receiving the same estrogen treatment In addition EBF was measured in pregnant animals 6 at the 16th–18th day and 6 at the 40th–45th da of pregnancy

The trachea was cannulated under light ether anesthesia with the animal fixed in supine position and the anesthesia was then continued by a mixture of 40 O_2 and 60 N_2O containing 4 6 vol fluorene (Fluoromar®) in an open system via the tracheal Y-cannula of the spontaneously breathing animal With the help of a human otoscope an 80 mm long blunt hypodermic needle (size 21 G) was inserted into the uterus through vagina and cervix so that the tip of the needle extended 5 15 mm beyond the external os, the position of th tip in one of the uterine horns being checked after the experiment Throu h the needle 0 1 ml of a ^{133}xenon solution (30 μCi dissolved in 0 9 saline) was injected after which the needle was flu ed with 0 1 ml of 0 9 saline A second experiment was carried out on the same animal 15 min after the first injection.

In the animals studied at late pregnancy a further double experiment was performed in order to measure also the ^{133}xenon clearance by the fetus-placenta unit after a small laparatomy 0 1 ml xenon-saline solut n was injected via a 6 G needle through the uterine wall into the amniotic cavity The cl-arance of the ^{133}xenon radiation in either type of experiment was recorded externally over the abdomen by a scintillation detector (opening diameter 4 cm) and a ratemeter (Meditronic) connected to an x t recorder The time constant was set at 5 s and the pulse height discriminator at 81 KeV

After completing the cl-arance curves the animal was decapitated and the entire uterus d sected ou freed from loose connecti e t ssue and weighed (after removal of the fetus and placenta in the pregna t animals)

The time required for radio ctivity to become halved ($T_{\frac{1}{2}}$) was calculted by transferring values (amou of radioactivity measured at every 30 s f 10 min after the injection) from the clearan e curve to semi logarithmic paper $T_{\frac{1}{2}}$ values above 0 min were rejected The blood flow was calculated as 100 (ln $T_{\frac{1}{2}}$) 0 7 ml 100 g min (Andersen and Ladefoged 1967) Only monoexponential clearance curves were used in the EBF determinations Both of the flow alues obtained for each exper ment were used in the calculations and Student s *t* test was applied to determine differences between mean alues

An add tional 10 guinea pigs were used for determination of the size of the uterine cavity and the mode of intraluminal distribution of injected rad oacti e solution Vaginal smears were prep red and sta ned in hemato yline-eosin for determination of cycl c phase after which the animal was killed b ceca at n under ether anesthesia One piece comprising the entire circumference was cut from each uter ne h rn 15 mm below the tubal-uterine junction and frozen in propane propylene (kept at the t mperature of l quid n trogen) to the chuck of a cryostat Sections of 10 μm thickness were cut trans ersely at 5 C and stain-d as above for l g t microscopy For stud es of the intraluminal d stributio the a ma s were anesthesized and 0 1 ml of a methylene blue solution was injected into the uterine ca ity in the same manner as in the flow e periments They were killed by decapitation either immediately after comple of the t jection or 10 min later The abdom nal ca ty was exposed and the cervix and uterine horns re gently cut open lo gitudinally i spected and photographed

Results

The cavity in the uterine horns was always slit-shaped (Fig 1a) The maximum thickness of the wall was 1 5 mm in the proestrus stage (Fig. 1a) and 2 mm during estrus The



Fig 1 (a) Cryostat section of gunınea pig uterus in the proestrus stage demonstrating the slit shaped lumen and the thickness of the endometrium (1 15 mm) and myometrium (0 35 mm) The scale is 0 5 mm (b) Guinea p g uterus cut open immediately after injection of 0 1 ml of a coloured (methylene blue) solution via vagina and cervix to illustrate distribution in the uterine cavity of intraluminally applied rad oactivity The entire horn into which the tip of the injection needle was located is well stained whereas the solution has reached only into the most proximal part of the contralateral horn. The scal is 10 mm

myometrium comprised approximately 0 4–0 5 mm (Fig 1a) in all stages studied The injected solution distributed (Fig 1b) immediately in the entire cavity (about 40 mm long) of that horn in which the tip of the needle was located The same picture was seen 10 min after the injection The solution only reached into the most proximal part of the cavity in the contralateral horn (Fig 1 b)

The results of the EBF measurements and changes in uterine wet weight are summarized in Fig 2 There was a significant ($p < 0\ 005$) increase of flow both in the estrogen treated but non operated animals (Groups 1 *vs* 2) and in the estrogen treated oophorectomized animals (Groups 3 *vs* 4 $p < 0\ 01$) The statistical significance is increased ($p < 0\ 005$) when all animals not receiving estrogen (Groups 1 and 3) are taken together and compared with all estrogen treated animals (Groups 2 and 4) However no difference in EBF (per g tissue) was shown between normal untreated and oophorectomized untreated animals (Groups 1 *vs* 3) or between normal estrogen treated and ovariectomized estrogen treated animals (Groups 2 *vs* 4)

Pregnancy in early stages (17–18 days *post coitum*) caused a slight though non significant increase in EBF (in the parietal decidua) During late pregnancy (40–45 days) the flow was markedly reduced in comparison with the values from early pregnancy ($p < 0\ 001$) The EBF at this stage was even about 50 per cent lower ($p < 0\ 001$) than that recorded in non pregnant uterus

In attempts to compare flow in the fetus placenta unit with that of the parietal decidu

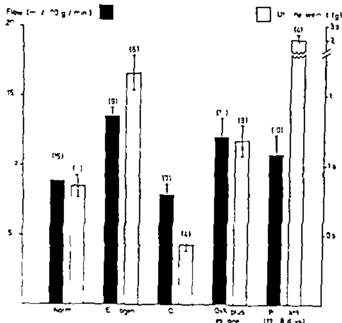


Fig 2 Variations in flow (clearance of intraluminal uterine administration of ^{133} xenon) and uterine wet weight under different conditions Mean ± S E, number of determinations within parenthesis Estrogen = 0 5 μg of 17β-estradiol benzoate s c daily during a week OvX = bilateral oophorectomy 3 weeks before the experiment

during late pregnancy (40–45 days *post coitum*) radioactivity was injected into the amniotic cavity after finishing the two measurements by intraluminal application As can be seen from Table I the mean flow in the fetus placenta unit was 3 times greater than in the parietal decidua ($p < 0.001$)

Discussion

Although no attempts were made to measure changes in blood pressure cardiac output blood gases etc in the guinea pigs the experiments were carried out under standardized conditions and the reproducibility checked by performing a large number of measurements. Fluroxene was chosen as anesthetic owing to its slight cardiovascular effects (Morris 1977) It was considered important to provide free airways with good spontaneous breathing by a tracheostomy a normal and constant pCO_2 level is then easier to maintain (altered pCO_2 affects cardiac output) and an adequate ventilation fascilitates the clearance of the xenon from the blood (thus eliminating recirculation of the radioactivity)

Secher *et al* (1973) and Einer Jensen and Juhl (unpublished) recently introduced the resorption of ^{133} xenon from the uterine lumen as a measure of the local blood flow in rats dogs and humans The technical advantages when using the intraluminal application technique on small animals such as rats were confirmed in the present study on guinea pigs. Resorption of administered radioactivity from the uterine cavity involves two components first distribution within the uterine cavity and diffusion through the epithelial cells and adjacent connective tissue and, second diffusion into and through the endothelial cells of the capillary walls After the intraluminal application of the xenon solution the uterine cavity

TABLE 1 Blood flow in the parietal decidua (^{133}xenon deposited in uterine lumen) and clearance of intra amniotically administered ^{133}xenon (flow in the fetus placenta unit) in pregnant (40–45 days) guinea pigs

Number of *fetuses*	Flow parietal decidua *ml/100 g/min*	Flow fetus placenta *ml/100 g/min*	Flow ratio uterus/fetus placenta
3	3 2	25 4	4 9
	4 7	13 4	
1	2 6	6 1	3 5
	2 6	11 8	
2	6 4	16 1	2 3
	6 5	13 8	
4	3 3	7 4	3 6
	2 5	13 4	
4	6 4	17 9	2 4
	8 6	18 6	
4	2 3	3 1	1 9
	2 3	5 5	
Mean ± S E.	4 3 ± 0 63	12 7 ± 1 85	3 1 ± 0 44

was exposed to radioactivity during 1 min after which the cavity was flushed with saline Using a stained marker it was found in model experiments that the administered solution distributed immediately after the injection in the entire cavity of the horn in which the injection needle was placed and that the solution only reached into the most proximal part of the contralateral horn during the time needed to obtain the clearance curves The uterine lumen is slit shaped th slit being 50 μm wide Assuming that the lumen contains a secretory fluid it means that the radioactivity in the first resorption component must diffuse approximately through a 25 μm liquid layer and the epithelium measured to be 20 μm thick The diffusion coefficient (D) is close to 10 ^{5} cm /s (Weast 1971) According to Einstein s equation where diffusion distance (r) = 2D t (t = diffusion time) applied to the exponential diffusion through a sheat (Davson 1970) the time needed for 50 equilibrium becomes 0 38 s At the end of the 1 min exposure to radioactivity (immediately before recording of the xenon clearance started) the zone of 50 / equilibrium calculated only with regard to diffusion and not perfusion will be located approximately 520 μm into the uterine wall i e in the endometrium Similarly the time needed to reach 50 % equilibrium at the endometrium myometrium border (calculated for 1 mm thick endometriums) is 9 1 min Therefore if also the perfusion process is included in the calculations it is apparent that the myometrial flow has not been included in the present measurements It should be mentioned that in determinations of uterine blood flow after intraluminal application in mice (unpublished observations) the clearance curves constantly consisted of two components—in mice the uterine wall is much thinner than in guinea pigs The clearance curves do not seem to have been influenced by passive diffusion of the xenon away from the area measured as evidenced by the practically horizontal curve obtained in pilot experiments involving recordings on dead animals

The mean EBF (8 9 ml/100 g/min) found in untreated guinea pigs was in the same order of magnitude as the flow values obtained in dogs after intraluminal (7 0 ml/100 g/min) and myometrial (8 5 ml/100 g/min) uterine application (Einer Jensen and Juhl 1974 and unpublished) *In humans the same routes of xenon administration (Secher, et al 1973)* gave a close relation between the flow values (intraluminal route = 0 9 × myometrial − 0 57 correlation coefficient = 0 89) The figures on guinea pig EBF are however about twice the flow values found after luminal application in rats (Einer Jensen and Juhl unpublished)

The result of an increased EBF following estrogen treatment confirms earlier observations on uterine blood flow in other animal species using different techniques (*cf* Greiss and Anderson 1970) and is in accordance with the higher flow during the estrous period of the ovarian cycle (Greiss and Anderson 1969) After oophorectomy there was no change in flow calculated per weight unit However the concomitant highly significant marked decrease in uterine weight indicates that there was a reduction in total flow measured in the uterus (*cf* Greiss and Anderson 1970) These experimentally induced changes in blood flow have been possible to detect also by measurements of uterine blood volume in mice using a radioiodine dilution technique (Brody *et al* 1974) It can be assumed that such changes in blood volume though are primarily representative of alterations in vascular capacitance function

During early pregnancy the results showed a tendency though non significant to an increased blood flow As the weight of the uterus however was doubled it means that the total blood flow measured was approximately doubled During late pregnancy the flow was significantly lower than that measured three weeks earlier which agrees with the pattern of changes in uterine blood flow reported by Csépli *et al* (1968) who also refer to other relevant work On the other hand a high flow rate is maintained in the fetus placenta unit indicating a regional heterogenity in the flow of the uterine vascular bed at least during late pregnancy

Supported by grant No 680-0383 A from the Ford Foundation New York

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Acta physiol scand 1975 93 104–112

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Ionic Requirements for Rapid Axonal Transport *in vitro* in Frog Sciatic Nerves

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Received 13 June 1974

Abstract

EDSTRÖM A *Ionic requirements for rapid axonal transport in vitro in frog sciatic nerves* Acta physiol scand 1975 93 104–112

The effects of K Na hypo- and hypertonicity on the synthesis and fast axonal transport of ^3H leucine-labelled protein were studied *in vitro* in the frog sciatic system. The methodology used made it possible to discriminate between effects on synthesis and transport of protein The preparation which consisted of the dorsal ganglia the sciatic nerve and the gastrocnemius muscle was placed in an incubation chamber The ganglia were incubated in standard Ringer containing ^3H leucine and the nerve was perfused with modified Ringer Perfusion of the nerve for 17 h with K free Ringer or Na free Ringer did not affect the rapid axonal transport of ^3H leucine labelled material from the ganglia along the nerve towards a ligature in front of which it accumulated Nor was the transport influenced by concentrations of K up to 68 8 mM In contrast concentrations exceeding 100 mM K partially inhibited the transport. Inhibition by ouabain (0 1 mM) was not prevented by elevating K to 30 mM Deviation from isotonicity towards a hypo- or a hypertonic medium partially inhibited axonal transport The transport inhibitory effects showed reversibility Experimental conditions which arrested the transport, were tested in separate experiments for effects on uptake of ^3H leucine into TCA soluble and insoluble ganglionic components K substituted for Na ouabain (0 1 mM) and hypotonic Ringer partially inhibited the amino acid uptake but also subsequent steps in the incorporation process, whereas only the latter was inhibited by hypertonic Ringer The results are discussed in relation to possible changes in energy metabolism

Results from studies on rapid axonal transport *in vivo* are accurately reproduced *in vitro* with the use of a technique designed for frog nerves (Edstrom and Mattsson 1972) which offers experimental approaches not possible in the living animal To understand the mechanisms of fast axonal transport it seems essential to know its relation to the ionic milieu It is also of importance to define suitable external conditions for future *in vitro* experiments on axonal transport

Results from *in vitro* studies on fast axonal protein transport in frog sciatic nerves suggest that Ca is not required for the maintenance of the transport mechanisms. The fast transport did not depend on the presence of external Ca or Mg whereas excess Ca^{2+} partially inhibited the transport (Edström 1974) Direct injection of EGTA a strong Ca^{2+}-chelator in the frog sciatic nerve failed to affect the rapid protein transport (Abe

et al 1973) There are only scattered data on the effects of Na^+ and K^+ on axonal transport Partlow *et al* (1972) did not find any difference in the rapid rate of cholinesterase in sciatic nerves in isotonic KCl isotonic NaCl or Ringer Solution In contrast removal of sodium from Krebs solution markedly inhibited the rapid transport of noradrenaline in the cat hypogastric nerve (Kirpekar *et al* 1973) In the present work the effects of monovalent cations and of hypo- and hypertonicity on rapid axonal transport in frog sciatic nerve were studied

Methods

The transport system Frogs *R pip ens* and at the end of the investigation *R temporaria* were used A preparation which consisted of the dorsal ganglia (no 8 and 9) the sciatic nerve and the gastrocnemius muscle was placed in frog Ringer solution in an incubation chamber with three compartments (A B and C) The parts were separated from each other by S licone grease barriers

^3H leucine was added to the ganglionic compartment (compartment A) which made it possible to follow the transport of labelled proteins from the ganglia, along the sciatic nerve (compartment B) towards the muscle (compartment C) The two preparations from the same an mal were used in each experiment One preparation served as a control and the other was subject d to various experimental conditions A ligature was placed on the nerve (compartment B) 30 mm from the ganglia The preparations were incubated for 17 h at 18 C Proteins synthes zed in the ganglia are during these conditions transported within the axons in anterograde d rection at a rate of 127±10 mm per day (Edstrom and Mattsson 1972 Edström and Hansson 1973) After treatment with trichloracetic ac d (TCA) the distribution of radioactivity along the nerve was determined as described previously (Edström and Mattsson 197?) In most experiments the transport function was measured by determining only the amount of TCA insoluble radioactivity which accumulated in a 4 mm nerve segment immediately in front of the ligature

In separate experiments ganglia were assayed for incorporation of ^3H leucine into TCA insoluble and soluble components After incubation the ganglia were washed twice ‗0 sec each time with Ringer before extraction of TCA-soluble radioactivity (Andersson t al 1972)

TCA insoluble samples were dissol ed in soluene 350 (Packard) in a 0 55 Permablend III (Packard) solution and were analysed for radioactivity with a Packard TriCarb (3375) liquid scintillation spectrometer TCA soluble extracts were counted in Bray s solut on (Bray 1960)

Chem cals Aqueous solut ons of L 4 5-^3H leucine (46–58 Ci/mmole 1 0 mCi ml) and freeze-dri d cycloleuc ne (carboxyl- C)(53 mCi mmole 405 μCi/mg) were purchased from the Radiochemical Centre Amersham England The standard Ringer had the following composition in mM NaCl 111 ? KCl 1 9 CaCl 1 1 $MgSO_4$ 1 6 NaH_2PO_4 0 45 Na HPO 2 6 and glucose 5 5 When the concentration of K^+ w s changed isotonicity was mainta ned by altering the molarity of Na Howe er in the K free solution KCl was om tted without changing the NaCl concentration Na free solution was prepared by com pletely replacing NaCl by isoosmolar choline chloride (or sucrose) and the sod um phosphates by the correspond ng potassium phosphates In some experiments hypertonic (00% R nger) and hypotonic (50 % Ringer) Ringer solutions were used All components (except glucose) of standard Ringer were than present at double and half concentrat ons In all various solutions the glucose concentration of standard R nger (5 5 mM) was kept constant The solut ons were gassed with O before use and the pH was adjusted to 7 4 by adding small amounts of HCl

Results

Transport in K^+ and Na free Ringer The preparations were incubated for 17 h at 18 C with ^3H leucine present in the ganglionic compartment (compartment A) At the beginning of the incubation a ligature was placed on the nerve (compartment B) 30 mm from the ganglia Standard Ringer was used in the ganglionic compartment During normal conditions labelled proteins of ganglionic origin are transported within the axons at a rate of 127±10

TABLE I Preparations were incubated for 17 h at 18 C Compartments A contained each 0 μCi ³H leucine (51 Ci/mmole) in 1 ml standard Ringer Paired preparations were used Compartments B were perfused with standard and test Ringer respectively TCA insoluble radioactivity which accumulated in a 4 mm nerve segment immediately in front of the ligature was measured Results are expressed as mean values of paired preparations ± S E M P values obtained by use of a two tailed t test based on percentage differences of paired samples N S = not significant (>0.)

	Number of expts	Counts/min/nerve segment proximal to ligature	Per cent of control	P
Control	20	9 112 ± 988		
K free Ringer	20	8 124 ± 998	95 5 ± 9 0	NS
Control	4	8 5.0 ± 652		
Na free Ringer	4	9 548 ± 1 313	110 4 ± 7 4	N S

mm per day at 18 C and accumulate in front of the ligature (Edstrom and Mattsson 1972, Edstrom and Hanson 1973) The transport rate and the rate of protein synthesis in the ganglia are maintained unchanged during 17 h and the amount of labelled protein accumulates linearly (Edstrom and Mattsson 1972 Edstrom and Larsson unpublished) If the nerve in compartment B was perfused for 17 h with K^+ free or Na free Ringer (replacement with choline⁺) the transport was unaffected (Table I) In two experiments (not shown) Na^+ was replaced with sucrose instead of choline⁺ Again there was no effect on the transport The barriers between the compartments have been shown to effectively hinder the movement of solutes Any accidental leakage of K^+ or Na^+ would furthermore be rapidly eliminated by perfusion through which the medium surrounding the nerve is kept unchanged

Transport in K high Ringer At a K^+ concentration of 68 8 mM (exchanged for equimolar amounts of Na) there was no effect on the transport (Table II) However at 90 mM K there was some small inhibitory effect in both of two experiments performed (not shown). At 113 1 mM K the amount of accumulated radioactivity was reduced to about 46 % of that in the control (Fig 1 Table II) whereas the protein synthesis in the ganglia kept in standard Ringer during the experiments was unaffected

TABLE II Except from test media experimental conditions as in Table I In 6 of these expts the nerves were cut in consecutive 4 mm segments and the distribution of radioactivity has been plotted in Fig 1 * The difference between 0 1 mM ouabain and 0 1 mM ouabain in 10 mM K Ringer is not statistically significant

	Number of expts	Counts min nerve segment proximal to ligature	Per cent of control	P
Control	4	9 4 4 ± 171		
68 8 mM K Ringer	4	8 61 ± 036	9 7 ± 1 2	N.S
Control	10	10 511 1 648		
113 1 mM K Ringer	10	4 799 ± 1 076	45 8 ± 11 1	0 005
Control	8	5 431 + 878		
0 1 mM ouabain	8	1 508 139	37 1 ± 10 0 *	< 0 005
Control	7	5 509 + 1 017		
0 1 mM ouabain in 10 0 mM K Ringer	7	1 909 ± 466	35 4 ± 19 6	0 005

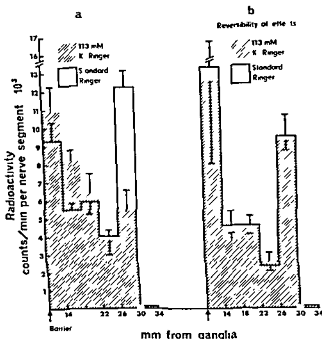


Fig 1 Distribution of ^{3}H leucine labelled TCA insoluble material in the nerve after incubation at 18 C in 113 1 mM K Ringer (see Methods) and in standard solution The position of the ligature is shown (X) Each bar represents the mean of 6 (1 a) and of 4 (1 b) expts The vertical lines indicate the S E M (*a*) One preparation was used as a control. The other from the same frog was perfused with 113 1 mM K^+ Ringer for 17 h To the compartment A 20 μCi ^{3}H leucine (51 Ci/mmole) in 1 ml standard Ringer was added (*b*) The two preparations from the same animal were used one serving as a control and the other as a test preparation Compartments B of the control and the test preparation were perfused with standard and with 113 1 mM K^+ Ringer respectively After 6 h the 113 1 mM K^+ Ringer was replaced by standard Ringer and 20 μCi ^{3}H leucine (46 Ci/mmole) in 1 ml Ringer was added to the compartment A of control and test preparations After another 17 h the nerves were analysed

To test if the inhibitory effect by 113 1 mM K^+ was reversible one preparation was incubated for 6 h with 113 1 mM K^+ Ringer in compartment B The control from the same animal was incubated simultaneously in standard Ringer Subsequently the K^+ high Ringer was replaced by normal Ringer and ^{3}H leucine was added to compartment A of the test and the control preparation After incubation for another 17 h the nerves were analysed The distribution of TCA insoluble radioactivity was similar in experiments and controls showing the reversibility of the inhibitory effect by 113 1 mM K^+ Ringer (Fig 1)

Ouabain (0 1 mM) has previously been shown to partially arrest rapid axonal transport in the present system (Anderson and Edstrom 1973) In the present study the ouabain effect was not significantly prevented by elevating external K^+ to 30 mM (Table II) which suggests that the ouabain effect is not directly mediated by inhibition of the Na^+–K^+ transport system This is also supported by the absence of effects by K^+ free or Na^+ free Ringer To test if the inhibitory effect by ouabain was reversible two experiments were run with 1 0 mM ouabain and two with 0 1 mM The experiments were designed as described above for testing the reversibility of effect by 113 1 mM K^+ Ringer The transport inhibitory effect

TABLE V Conditions as described in Table IV except that 8 μCi ^{14}C-cycloleucine (53 mCi mmole) in 1 ml Ringer was used Results represent the mean of 4 experiments

	TCA soluble radioactivity counts/min/ganglionic sample	Per cent of control	P
Control	27 385 ± 2 040		
113 1 mM K Ringer	14 185 ± 879	5 4 ± 4 1	< 0 005
Control	.8 884 ± 1 647		
0 1 mM ouabain	9 807 ± 615	34 1 ± . .	< 0 005

solution supplemented with 0 23 osmolar sucrose depressed the protein synthesis more than 200% Ringer *i e* increased concentration of not permeable non ionic solutes was more effective than increased concentration of permeable ionic solutes

Discussion

Rapid axonal transport in frog sciatic nerves did not show dependence on either medium K^+ or Na^+ Kirpekar *et al* (1973) reported that the appearance of noradrenaline fluorescence after occlusion of the cat hypogastric nerve required extracellular Na^+ Others have shown that in a medium of low Na^+ the loss of endogenous noradrenaline is significantly increased (Yessaian *et al* 1971) However the inhibitory effect by Na free medium observed by Kirpekar *et al* (1973) was considered to be at the level of axonal transport Whereas the present results show that rapid axonal transport of protein in the frog sciatic nerve does not require external Na^+ or K the importance of intracellular Na and K can not be excluded The cations may be very slowly removed from the nerves a possibility which is under investigation It is evident that rapid transport in frog sciatic nerves is independent of the transmembrane potential Impulse conduction in the frog sensory axons exposed to 45 mM K Ringer was abolished within 45 min whereas axonal transport of protein in the nerves was maintained unchanged (Andersson and Edstrom 1973) The absence of transport effects by a considerably higher K concentration (69 mM K) in the present study supports this

Potassium substituted for sodium ion in the Ringer solution the presence of ouabain (0 1 mM) hypo- and hypertonicity were experimental conditions which partially and reversibly arrested rapid axonal transport in the system used There are reasons to believe that these effects are secondary to depressed energy metabolism which will be considered next

At the presence of greater than 100 mM K the transport was inhibited The effect was reversible and not due to tissue damage Replacing sodium chloride by potassium chloride in the incubation medium of brain slices among other effects (see below) leads to uncoupling of phosphorylation and a depressed ATP availability (Heald 1960) Rapid axonal transport is closely dependent on energy from ATP and fails when the ATP generation is blocked (Ochs 1972) The present results show that protein synthesis which also depends on the supply of ATP was inhibited by 113 1 mM K Ringer The latter effect is likely to be at the level of active membrane transport of amino acid but also of some other step in the

incorporation process It is known that ($Na^+ + K^+$)-ATPase is partially inhibited by very high K^+ (Skou 1964) which could contribute to the inhibitory effect on amino acid uptake In contrast to the present results Partlow *et al* (1972) reported the absence of effects by 110 mM K^+ on rapid axonal transport of cholinesterase in isolated frog nerve segments The reason for the differences in the two investigations in which axonal transport was resolved with different techniques is not evident

Partlow *et al* (1972) also reported the absence of effect by a very high ouabain concentration (10 mM) whereas 0 1 mM ouabain caused 50% inhibition of axonal transport in the present investigation It is obvious that the latter effect is not due to membrane depolarization as such in this case by inhibition of the ($Na^+ + K^+$)-ATPase activity The ouabain effect was not abolished by raising the medium K^+ concentration and the transport did not show dependence on external Na^+ or K^+ Ouabain and other cardiac glycosides probably affect more enzyme systems than ($Na^+ + K^+$)-ATPase alone and have been shown to alter energy metabolism in several ways (Rolleston and Newsholme 1966) Among other effects stimulation (Lefevre *et al* 1970) but also depression of respiration in brain slices by ouabain have been reported (Whittam 1962) In the present study 0 1 mM ouabain partially inhibited rapid axonal transport but also protein synthesis The latter effect seems to comprise not only inhibition of amino acid transport but also of some later step of protein synthesis This conclusion is in accord with observations by others (Kypson and Hait 1971) Kirpekar *et al* (1973) who studied the axonal transport of noradrenaline *in vitro* in the cat hypogastric nerve did not observe any transport effect after treatment with 0 1 mM ouabain for 30 min The longer time of incubation (17 h) with 0 1 mM ouabain in the present experiments could possibly explain the discrepant results

The present results show that rapid axonal transport *in vitro* does not depend on any specific external monovalent cation whereas deviation from isotonicity partially arrested the transport The latter is perhaps not surprising Energy requiring processes such as melanin aggregation in melanophores (Novales 1971) cell division in mammalian cell cultures (Wheatley and Angus 1973) and protein synthesis (Austin *et al* 1970) are inhibited by hypertonicity It is interesting to note that the two former processes like axonal transport are supposed to be dependent on the function of microtubules Effects by hypertonicity on colour changes and on ultrastructure in skin melanophores suggest bulk movements of water in connection with melanin movements (Novales 1971) an aspect which has so far been little considered in association with axonal transport The reasons for the inhibitory effects on axonal transport may be several The protein synthesis in the ganglia was partially inhibited in corresponding hypo- and hypertonic solutions and one common reason for the inhibitory effects on axonal transport and protein synthesis may again be impairment of energy metabolism

Standard Ringer plus sucrose inhibited the transport much more than 200 Ringer which indicates that the effect was due to increased tonicity rather than to increased ion concentration as such This is interesting since increased ion concentration is considered to damage the membrane of the frog sciatic nerve more than increased concentration of nonionic solution (Pribor and Nara 1973) which suggests that the mechanism of rapid axonal transport is not intimately associated with membrane processes

The present work was supported by grants from Statens Naturvetenskapliga Forskningsråd (No 2535-8) and C B Nathorsts Vetenskapliga och Allmännyttiga Stiftelse Thanks are due to Mrs B Egner for her expert technical assistance

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Acta physiol scand 1975 93 113–118

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The Excitatory Action of Acetylcholine on Intradental Sensory Units

By

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Received 18 June 1974

Abstract

HAEGERSTAM G L OLGART and L EDWALL *The excitatory action of acetylcholine on intradental sensory units* Acta physiol scand 1975 *93* 113–118

In order to test the hypothesis that ACh mediates the transmission of pain stimuli from d ntin to sensory intradental nerve endings the following experiments were performed

Intradental nerve impulses were recorded by means of low impedance electrodes inserted in dentinal cavities in the tooth of the cat An air blast proved to be an efficient physical stimulus to excite the intra dental nerves Local application of acetylcholine caused a similar response This response to acetylcholine was followed by a transient blockage to repeated applicat on The response to acetylchol ne could be blocked by d tubocurarine atropine succinylcholine and hexamethonium administered locally In contrast, the response to physical stimuli (air blasts) could not be blocked by these drugs Moreover during the period of depress on following acetylcholine the preparation responded to physical stimuli These findings suggest that acetylcholine is not a med ator in the intradental pain transmission provoked by physical st muli

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Various studies have suggested that acetylcholine (ACh) plays a role in the transmission of pain Among the findings is that ACh causes pain when applied to the blister base (Keele and Armstrong 1964) or the exposed dental pulp (Anderson and Naylor 1962) Scott (1966) has recorded nerve impulse activity from deep dentinal cavities in the cat caused by local application of ACh Furthermore acetylcholine esterase has been found in the dental pulp (Avery and Rapp 1958 Pohto and Antila 1969 Arwill and Lilja 1972) taken to indicate the presence of ACh (Avery and Rapp 1958) In addition some kind of junction between nerve like structures and odontoblasts has been observed in human dentin (Frank 1966 Arwill 1967 Roane *et al* 1973) as well as in the dentin of the cat (Arwill *et al* 1973) These observations might indicate that the involvement of ACh in dental pain is as a transmitter in a synaptic junction as suggested by Avery and Rapp (1958) Similar mechanisms have been suggested for other types of peripheral sensory receptors (Koelle 1962)

The aim of the present study was to investigate the role of ACh in pain mechanisms in the tooth

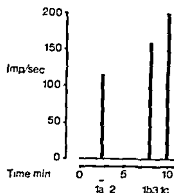


Fig 3 The effect of hexamethonium on intradental nerve response upon stimulation by airblast and acetylcholine 1 Air blast Hexa methonium 40 mM 3 Acetylcholine 5 5 mM

1962) In some experiments of the present study the cavities were made shallow In these cases application of ACh or KCl produced responses of low impulse frequency and long *latency between the application and the response or failed to excite the sensory units at* all A probable explanation of these findings is that the most peripheral sensory units in dentin are located in the inner dentin (Arwill *et al* 1973) Thus a shallow cavity results in greater diffusion distances to excite the neurons In addition the concentrations of ACh and potassium chloride used in this study were ten to twenty times that used by Anderson and Naylor These considerations may explain the discrepancy between the results obtained *in the studies by Anderson and Naylor and Brannstrom and those in the present study*

The excitatory action of ACh on the intradental neurons observed in the present study may be due to a direct influence of ACh on the nervous membrane or an indirect effect caused by a change in pulpal blood flow Such a change in blood flow is possible since it is well known that ACh can relax vascular smooth muscle In the dental pulp ACh has been shown to change the intrapulpal pressure (Kawamura Kato and Kato 1967 Lemay Laliberté and Simard Savoie 1972) and in our preparation this action may result in excitation of the sensory units The influence of ACh on pulpal blood flow has recently been investigated in this laboratory (Edwall Olgart and Haegerstam 1973) These studies showed

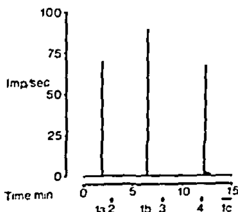


Fig 4 The effect of atropine on intradental nerve response upon stimulation by air blast and acetylcholine 1 Air blast Atropine 3 5 mM 3 Acetylcholine 5 5 mM 4 Potassium chloride 135 mM

that under the same experimental conditions as in the present study pulpal blood flow was not changed by close intra arterial infusion of ACh

Based on studies on the blister base preparation Keele and Armstrong (1964) reported that the pain response upon local application of ACh could be blocked by drugs such as atropine d tubocurarine hexamethonium and succinylcholine In the present study we found that these drugs blocked the intradental nerve impulse response to ACh These blocking agents have different modes of action atropine is a competitive blocking agent to ACh at the muscarinic receptor sites d tubocurarine competitively blocks the ACh receptor sites in the neuromuscular junction succinylcholine is known as a depolarizing agent at this junction and finally hexamethonium blocks competitively the ACh receptor sites in autonomic ganglia

Thus the excitatory response to ACh the period of desensitization and the blocking action of drugs with different mode of actions indicate the existence of an unspecific effect of ACh on the intradental sensory unit These findings are not compatible with the theory that there is a cholinergic synapse transmitting various dentinal pain stimuli from the odontoblast to the sensory nerves as proposed by Avery and Rapp (1958) The experiments with air blasts were performed in order to elucidate whether such a cholinergic receptor plays any significant role in the transmission of physical stimuli

It is clinically well known and experimentally verified (Brannstrom 1966) that an air blast on the exposed dentin is a potent pain stimulus In the present study air blasts evoked bursts of impulses However this response was not influenced by the desensitization which followed a previous application of ACh Furthermore the response was not influenced by atropine d tubocurarine hexamethonium and succinylcholine in doses sufficient to inhibit the response to ACh

These findings indicate that intradental nerve impulses evoked by physical stimuli (air blasts) are not mediated by a cholinergic mechanism and strengthen the argument against the proposed existance of a cholinergic synapse between the odontoblasts and sensory nerve endings

Based on experiments on dermal sensory nerves Douglas and Ritchie (1959) suggested that sensitivity to ACh is a general property of sensory nerve endings regardless of their functional specialization The results obtained in the present study are in agreement with their suggestion

This in estigation was supported by grants from the Swedish Medical Research Council (B74-?4X 816-09) the Swedish Association of Medical Research Karolinska Institutet, Stockholm and Astra Läkemedel AB Södertälje

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Acta physiol scand 1975 93 119–128
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Cerebral Units Activated by Tactile Stimuli via a Ventral Spinal Pathway in Monkeys

By

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Received 26 June 1974

Abstract

ANDERSSON S A S FINGER and U NORRSELL *Cerebral units activated by tactile stimuli via a ventral spinal pathway in monkeys* Acta physiol scand 1975 *93* 119–128

Extracellular microelectrode recordings were made in the forelimb and/or hindlimb areas of the cerebral post-central gyri of 7 monkeys before and/or after acute dorsal hemisections of the cervical or low thoracic spinal cord The animals were anaesthetized with either pentobarbitone sodium or chloralose Among the units which were encountered after the lesions were 114 which could be influenced from the periphery 38 units discharged to light tactile stimulation of small receptive fields and had a topographical organization like that observed in the intact animal The remaining 76 units could be activated by peripheral electrical stimulation and/or by tapping squeezing or light pinching No units were found which specifically were activated by noxious stimuli The two groups of cortical units responded with latencies only slightly longer than those obtained in the intact preparation In the light of previous findings these electrophysiological data suggest that unlike carnivora primates may possess the ventral spinal somatosensory pathway traditionally postulated on the basis of neurological material

For more than one hundred years experiments on animals and human case reports have been showing that transection of a single ascending spinal pathway does not totally abolish the sense of touch from below the level of the lesion (*vide* Petrén 1902) The individual reports on the effects of circumscribed spinal cord lesions have been widely different and sometimes contradictory Nevertheless the data have led to acceptance of the theory that more than one spinal pathway has the potential for transmitting information about peripheral tactile events to higher nervous structures (*vide* Peele 1954)

The results of electrophysiological experiments have had similar implications It has been found for example that neurones of the cerebral somatosensory areas in the anaesthetized cat can be activated by tactile stimulation of restricted peripheral fields on the body surface as long as at least one of two dorsally located ascending spinal pathways remains intact *i e* the dorsal funiculus or the spino-cervical tract (Andersson 1962 Levitt and Levitt 1968) No comparable cortical activation was observed when all pathways ascending in the dorsal half of the cat s spinal cord had been transected (*loc cit*)

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Analogously several spinal pathways appear to project with short latency to the first cerebral somatosensory area of the monkey (Gardner and Morin 1957 Andersson Norrsell and Norrsell 1972, Eidelberg and Woodbury 1972) As far as can be judged from the *recording of surface evoked potentials in the anaesthetized monkey before and after spinal* lesions however the most important pathway remaining after transection of the dorsal funiculi is one located ventrally in the spinal cord (Andersson *et al* 1972)

The present experiments were made in order to establish whether in contrast to previous findings on the cat neurones of the first somatosensory area of the anaesthetized monkey can be activated by light tactile stimulation of restricted peripheral fields after transection of the dorsal half of the spinal cord These experiments extend findings presented in a preliminary report (Andersson and Norrsell 1973)

Methods

The experiments were made on 6 cynomolgus (*M Fascicularis seu Irus*) and a grivet (*Cercopithecus Aethiops*) monkeys of 3 0–4 0 kg b wt Four of the animals were anaesthetized with pentobarbitone sodium (Nembutal Abbott) whereas the others were anesthetized with D gluco-Chloralose Successive doses of the chosen anaesthetic were administered throughout each recording session to keep the animals under a moderate level of anaesthesia All the monkeys received Gallamine jodidum (Flaxedil Rhodia) and were given artificial respiration The expiratory P_{CO_2} was monitored and maintained at physiological levels at all times, and body temperature was held between 38 and 39 C

For 3 animals surgery consisted of a cervical laminectomy which gave access to spinal segments C4–C5 for lesion placement 4 other animals received laminectomies and lesions at the low thoracic level. No spinal cord surgery was performed in one monkey The spinal cord lesions were made under a binocular dissecting microscope with watchmaker s forceps The lesions consisted of transection of the dorsal part of the spinal cord to the depth of the central canal thus including the dorsal funiculi and the dorsal halves of the lateral funiculi Part of the sensorimotor cortex was exposed in all animals by a unilateral craniectomy and removal of the underlying dura Photographs of the exposed cortex were made and developed immediately to provide convenient guides for the accurate placing and replacing of the electrodes

Extracellular unit recordings were accomplished with the aid of a closed chamber system which diminished the pulsation of the cerebral cortex (Andersson and Källström 1971) An attempt was made to mount the chamber in a way that permitted the electrodes to penetrate the cortex perpendicularly The electrodes were filled with 3 M NaCl and had a resistance of 1 3 MΩ. The potentials were fed into an amplification system with a long time constant and were displayed on a cathode ray oscilloscope and through an auditory channel Relevant events were registered photographically during the experiments.

Hair brushing and manipulation of joints and peripheral structures served as stimuli while a microelectrode was being lowered slowly into the cortex As soon as a unit was isolated the entire surface of the animal was explored systematically to establish the modality and receptive field of the unit If light mechanical stimuli (blowing, camel hair brush or blunt probe) were ineffective surface and subcutaneous structures were exposed to stronger stimuli 0 1 ms square wave pulses were delivered through small bipolar needle electrodes that were inserted into the centre of a receptive field or close to the peripheral nerve in order to obtain latency measurements.

At the end of each recording session the animal was perfused with physiological saline followed by 10 buffered formalin olution The region of the spinal cord containing the lesion was removed and embedded in paraffin after further formalin fixation *in situ* Reconstructions of the lesions were made from serial sections which were stained according to Klüver and Barrera (1953) The dorsal half of the spinal cord was found to have been transected as planned in all cases

Results

The major purpose of the present investigation was to examine single units of the postcentral gyrus after lesion of the dorsal part of the spinal cord A number of the monkeys

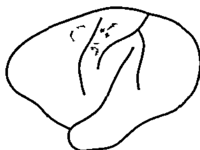


Fig. 1 Schematic representation of the cortical areas in which micro electrode penetrations were made after dorsal hemisections of the spinal cord Each pattern represents the penetrations made in a single monkey

underwent unit recordings just prior to the transections however in order to confirm recording loci and to obtain sufficient data for pre lesion/post lesion comparisons The general distribution of the post lesion penetrations in the post-central gyri of the different animals is illustrated in Fig 1 This diagram shows a region of the pre-central gyrus in which recordings were made after this type of lesion in one monkey (see below)

After the spinal cord lesions 114 units which could be activated by peripheral stimulation were isolated in the post-central gyri Other cells were spontaneously active and could not be influenced from the periphery The units were collected in 73 penetrations The number of units per penetration excited from the periphery thus was considerably lower than the number (4–18) experienced prior to a lesion

Table I summarizes some of the basic characteristics of these cortical units This table does not include separate subsections for the different cytoarchitectonic areas or for the two varieties of anesthesia Potentially important differences might have been obscured by the limited amount of sample material Examination of the characteristics of the units failed to reveal any consistent variations however that could be attributed to either cytoarchitecture or the type of anesthetic Furthermore the type and number of cells per penetration did not appear to change as a function of whether the preparation involved

TABLE I Some characteristics of units found in the post-central gyri which were activated from peripheral fields located caudal to dorsal hemisections of the spinal cord The units were found in six different monkeys Values signify the median and the range

	Units activated by light touch from restricted skin fields			Units activated by other types of peripheral stimulation	
	Size of receptive field (cm)			Total number of units	Latency[a] (ms)
	Movement of hairs	Other tactile stimuli	Latency[a] (ms)		
Forelimb	1 (0 5–5) N = 8	1 (0 5–[illegible]0) N = 10	1[illegible] (9–15) N = 1[illegible]	N = 31	1[illegible] (8–[illegible]5) N = 15
Hindlimb	[illegible]5 (1–5) N = [illegible]	[illegible]5 (1–[illegible]0) N = 18	17 (13–25) N = 14	N = 44[b]	[illegible]0 (15–30)[b] N = 6

[a] Latency measurements were not obtained for all units.
[b] One unit could not be included in this tabulation This unit was activated 11–1[illegible] ms after electrical stimulation of the contralateral ankle and gave slowly adapting discharges to small changes in the angle of that joint

forelimb recording after a cervical cord lesion or hindlimb recording after a cervical or thoracic lesion (see Table I) For these reasons the aforementioned conditions have not been treated separately in the descriptions which follow

I *Comparison of unit activity before and after transection of the dorsal half of the spinal cord*

Adequate tactile or electrical stimulation in the approp iate p riph-ral field e cited a short latency mass discharge and intense unit activity in the post-central gyri of preparations with the spinal cord intact The cells possessed small contralateral receptive fields which were easily delineated In contrast the impression was one of partial denervation afte th lesions The mass discharge which was evoked by peripheral stimulation was markedly diminished and a large number of the units displayed a low or moderate spontaneous activity which could not be influenced from the periphery Although background units appeared to respond to peripheral stimuli it was sometimes difficult to isolate single units with discharges of sufficient amplitudes and stability to permit individual characterization.

Nevertheless 38 cells were identified which responded to low threshold tactile stimuli such as blowing or light touch and which had well defined receptive fields which frequently were in the range of 1 cm^2 (Table I) These cells were found under both nembutal and chloralose anaesthesia (Fig 3) and like all units which could be influenced with peripheral stimulation their topographical arrangement resembled that noted in the intact animal The latencies of these and other units which could be influenced from the periphery did not appear to be markedly longer than those of units recorded before the lesions

The upper part of Fig 2 shows 4 units which were encountered during one penetration with the spinal cord intact The dorsal half of the spinal cord was transected at Th 10 after the microelectrode had been retracted The electrode then was returned into the same track and 3 units were found at depths approximating those at which units had been identified earlier (Fig 2 lower half) No correlate of the fourth unit was present The units shown in the upper part of Fig 2 were activated by lightly touching the glabrous skin on the plantar surface and fired reliably to electrical stimulation of their receptive fie'ds with latencies in the range of 15 ms before the lesion They followed repetitive stimulation at frequencies of 10–20 s The three units that were found after the lesion were still excited by touching glabrous skin but they were slightly more difficult to activate and their discharges to adequate stimulation were more variable than before The exact boundaries of the receptive fields were now slightly more difficult to establish although the fields were not larger in size The latencies for these units were somewhat longer than they had been before the lesions and they could only follow repetitive electrical stimulation up to 4 Hz

II *Post-central units with small receptive fields that responded to light tactile stimulation*

38 of the 114 units which responded to peripheral stimulation following the lesions had small receptive fields and were excited by blowing on the hairs or by lightly touching hairy or glabrous skin They were activated from sites on the forelimb or hindlimb contralateral to the position of the recording electrode Most of the units were found on the distal parts of the limbs and had receptive fields which covered hairy skin only For some however

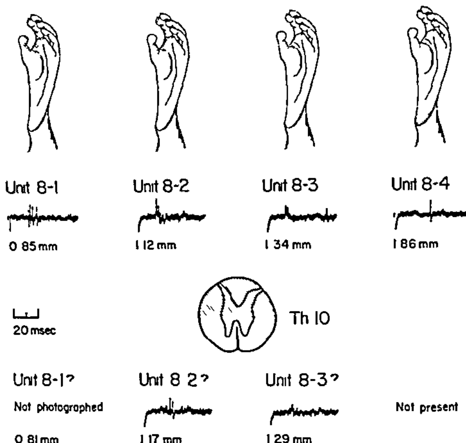


Fig Receptive f elds electrical responses and depths of un ts isolated during two microelectrode penetrations in the same track in the post-central gyrus The upper reco ds were obtained with the sp nal cord intact and the lower records were obtained d rectly after the lesion shown in the drawing The boundaries of the receptive f elds shown in the upper row did not cha ge after the transect on The units were found in a monkey anesthetized with chloralose

activation was accomplished by stimulating glabrous skin alone or hairy and glabrous skin together The receptive fields of these units tended to increase in size from distal to proximal peripheral loci as is usually noted in the intact preparation Yet some units with restricted skin fields were found close to or partially on the body Six units displayed prolonged activity to constant light touch but the rest of the units in this group were rapidly adapting None of these units showed inhibition

Fig 3 presents two units from this group Unit 2-2 was found with nembutal and unit 3-7 was found with chloralose anesthesia Both of these units responded to hair movements on the contralateral hand Both had very small receptive fields and displayed rapid adaptation to natural stimuli The latencies for the units were 10 and 11 ms respectively

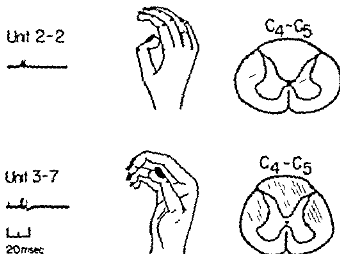


Fig 3 Electrical responses and receptive fields of two units activated by movements of hairs. These units were isolated in the post-central gyri of two monkeys following the spinal lesions reconstructed on the right Unit [illegible] was found with nembutal and unit 3 7 was found with chloralose anesthesia

III *Post central units activated by types of stimulation other than light touch*

The majority of the peripherally driven units (N ~ 76) were activated by types of stimulation other than light touch These cells typically were responsive to light pinching manipulating or tapping the limbs stretching the musculature or applying pressure to the deep tissues These stimuli were never strong enough to be considered noxious but noxious stimuli were applied if a unit responded to electrical stimulation but failed to respond to tactile stimulation No units in this group were activated by such stimuli

The discharge caused by a certain type of peripheral stimulation occasionally varied over time and it often was difficult to establish the location or size of the receptive fields of these units No units could be excited from the ipsilateral side of the body One or two action potentials were fired by most of the units in response to peripheral stimulation With repetitive natural or electrical stimulation however it was seen that these action potentials would not always follow the stimulus reliably even when the events were separated widely in time The responses evoked by electrical stimulation in many instances were more reliable than those evoked by adequate stimulation Four units of this group could not be activated by any type of natural stimulation but could be discharged via needle electrodes inserted into the skin

The spontaneous discharges of 5 units were inhibited by peripheral stimulation in one of the monkeys These cells were found in the hindlimb region in the border zone between cytoarchitectonic areas 1 and 3 Three of the 5 were inhibited by light touch in restricted fields of the contralateral hindlimb and were excited by manipulating or tapping another part of that limb The fourth unit could be inhibited by lightly pinching a small field and excited when the same stimulus was applied to a larger field (presumably in the skin) No excitatory field was found for the fifth unit This cell was inhibited when a region below the knee was touched lightly

IV *Further observations*

Two experimental manipulations deserve brief mention since they show that the monkeys were in sound physiological condition at the time of recording. First, recordings resembling pre lesion records were acquired when the forelimb projection was studied in an animal that had the dorsal part of the spinal cord transected at the thoracic level Penetrations in the cortical hindlimb area already had been made with results comparable to those described in the preceding paragraphs

Second hindlimb units were isolated without any difficulty in the precentral gyrus of one monkey with a lesion at Th 12 21 units were examined in this animal 10 could be activated by light tactile stimulation and 6 had receptive fields smaller than 5 cm^2 Almost all of these units were slowly adapting, and the latencies were in the 15–20 ms range The remaining 11 pre-central units were activated by stimuli other than light touch (*e g* pressure tapping, squeezing, joint rotation) and had large receptive fields Some of these fields were bilateral with part of one leg excitatory and part of the other leg inhibitory

Discussion

The type of information which can be transmitted to the first cerebral somatosensory area via pathways ascending in the ventral half of the monkey s spinal cord was examined in the present investigation It was found that some units of the post-central gyrus could be activated with light tactile stimuli (*e g* movement of hairs, gentle mechanical stimulation of glabrous skin) applied to small contralateral receptive fields located below the level of a dorsal hemisection of the spinal cord Similarly located and possibly identical units were observed in the same electrode track before and after spinal hemisection in one of the monkeys (Fig. 2) Hence the characteristics of this type of unit cannot be attributed to the spinal surgery *per se* Following the dorsal hemisections the cells showed latencies in the range of 12 and 17 ms from forelimb and hindlimb respectively Such latencies are comparable to those seen in surface evoked potentials of monkeys with intact spinal cords (Andersson *et al* 1972) These units did not have inhibitory fields although inhibition was observed in other cortical units after the lesions

The presence of this type of unit albeit in restricted numbers suggests that detailed information about tactile events can be transmitted to the forebrain via a ventral spinal pathway(s) in the primate A ventral projection does not appear to be present in carnivora insofar as similar cortical activation has not been observed in cats after spinal cord damage comparable to that made in the present investigation These experiments have involved recording from both the first (Levitt and Levitt 1968) and the second (Andersson 1962) area of the cat s somatosensory cortex

Andersson *et al* (1972) reported on the basis of evoked potential experiments that the major spinal pathway *other than the dorsal funiculus which projects to the first cerebral* somatosensory area of the monkey is one located in the ventral spinal quadrant contralateral to the afferent input It appears likely that the spinal pathway responsible for the earlier and the present results is the spino-thalamic tract This pathway consists of axons from both

cervical and thoracic levels which cross the midline and ascend in the ventral spinal quadrant Some of these axons terminate in the nucleus ventralis posterolateralis (VPL) of the thalamus of monkey and man (Bowsher 1957 Mehler Feferman and Nauta 1960) Although there is some controversy with regard to nomenclature a comparable pathway has not been found in the cat (Boivie 1971 Bowsher 1971) Furthermore experiments on monkeys have shown that the physiological properties of spino-thalamic neurones reaching VPL are such that they could be responsible for the characteristics of cortical units described here (Albe Fessard Levante and Lamour 1974) (Added in proof see also Bryan R N J D Coulter and W D Willis *Exp Neurol* 1974 42 574-586)

Following transection of the dorsal half of the spinal cord the number of units which could be activated from the periphery decreased markedly In addition many of the units which were found were not driven by well-defined peripheral stimuli It appears reasonable to expect a sizeable reduction in the number of units activated from the periphery after a transection that includes a major input to the forebrain somatosensory areas *i e* the dorsal funiculus (*vide* Norton and Kruger 1973) An apparent diminuity of the unit population could however materialize for other reasons Thus the spinothalamic tract may well be especially sensitive to anaesthetics (*vide* Albe Fessard *et al* 1974) The lesions would of course apart from interrupting ascending systems also interfere with any descending control system located in that part of the spinal cord The spino-thalamic tract like most other ascending spinal pathways has been found subject to supra-spinal control although the spinal trajectory of this control system has not yet been investigated (Coulter Maunz and Willis 1974)

Some recent observations by Dreyer *et al* (1974) appear to be relevant here These authors also combined lesion and electrophysiological techniques to examine the cortical projection of the spinothalamic pathway and noted that some units in the monkey s post-central gyrus could be activated by light tactile stimuli applied to restricted peripheral fields Dreyer *et al* (1974) did not emphasize this finding, and while their data appear to confirm our own it should be noted that the 2 expts are not directly comparable This is seen in the fact that these investigators used an unanaesthetized preparation and permitted 21 to 7. days for recovery from the spinal cord damage In addition the present lesions were symmetrical while those of Dreyer *et al* were assymmetrical and may have caused a different type of imbalance of the remaining ascending projection and descending control systems Differences such as these could account for the observation that their units with lemniscal properties were activated via the spino-thalamic tract only from the proximal parts of the limbs and or were located in the posterior part of the post-central gyrus No such limitations were observed in the present investigation

It has been argued that there is a pathway in the ventral part of the spinal cord with the potential for transmitting specific information about peripheral tactile events to the cerebral cortex of the monkey Comparable pathways appear to be located in the dorsal part of the spinal cord in carnivora and lesions which interrupt these dorsally located pathways in dogs and cats cause a marked deterioration in learned performance with tactile stimuli (Norrsell 1966 Kitai and Weinberg 19.8) The presence of a ventral pathway therefore could explain why lesions restricted to the dorsal half of the spinal cord do not result in

similar behavioural deficits in monkeys (Sandwald and Vierck 1968) and why observations of patients with partial damage of the spinal cord have traditionally led clinical neurologists to suggest the presence of such a pathway in man (*vide* Peele 1954) The results of any experiment involving partial lesions of the nervous system must be interpreted cautiously however and it may be premature to even speculate about the normal function of this pathway since its presence has been demonstrated by destroying tissue and hence *disrupting* the normal activity of the system It also must be remembered that the present experiments like most electrophysiological studies were concerned with differential activation of neu rones by stimuli received *passively* by the animals under standardized conditions Sensory systems might be more involved with other aspects of stimulation The major task of a sen sory system may be to assess the invariance of a given stimulus received *actively* by the organism under widely different conditions (Gibson 1966)

This work was supported by the Swedish Medical Research Council (project No 55 and ..857)
Miss Ewa Lignell and Mrs Kerstin Olson assisted in the experiments Mr Gosta Magnusson attended to the animals

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Acta physiol scand 1975 93 129–134
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Circulatory Effects Evoked by 'Physiological' Increases of Arterial Osmolality

By

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Received 1 July 1974

Abstract

JARHULT J J HILLMAN and S MELLANDER *Circulatory effects evoked by physiological increases of arterial osmolality* Acta physiol scand 1975 *93* 129–134

The effects of moderate arterial hyperosmolality (+20 mOsm/kg H_2O) produced by short term intravenous hypertonic infusion on vascular resistance in skin skeletal muscle intestine and kidney were analysed in the anesthetized cat Vascular resistance decreased in all four regions in response to the hypertonicity both before and after regional sympathectomy and the effects were not significantly altered by β adrenoceptor blockade Arterial blood pressure rose during the hypertonic infusion despite the decreased vascular resistance and an unchanged heart rate indicating an increased stroke volume and cardiac output Similar increases of arterial osmolality are known to occur in heavy exercise and in hemorrhage The present results may therefore suggest that blood borne hyperosmolality is a factor which can contribute to the overall cardiovascular adjustments in these situations

Investigations in recent years have demonstrated that hyperosmolality plays important roles in the control of the circulation by influencing vascular tone transcapillary fluid balance plasma volume etc (for ref see Mellander 1973) The osmolar control of vascular smooth muscle seems of special significance in skeletal muscle and glands in which a pronounced local tissue hyperosmolality develops during increased activity and contributes to the functional vasodilatation (Mellander *et al* 1967 Lundvall 1972 Lundvall and Holmberg 1974) Vascular smooth muscle in several other tissues is known to be responsive to experimental hyperosmolality produced by administration of hypertonic solutions to *the blood stream (e g Navar et al 1966 Gazitua et al 1971 Hauge and Bø 1971 Lundvall* 1972) The vascular effects of a generalized blood borne hyperosmolality as induced by intravenous hypertonic infusion have so far mainly been studied in response to drastic increases of osmolality (*e g* Muirhead *et al* 1947 Read *et al* 1960 Raizner *et al* 1973) which may be considered supraphysiological A moderate hyperosmolality (about 20 mOsm/kg H O above the control level) develop in th arterial blood during whole body exercise due to delivery of osmols from the active muscles (Lundvall *et al* 1972) and in hemorrhage due to glucose release from the liver (*e g* Jarhult 1973 1974) The present study was undertaken to analyse whether such physiological blood borne hyperosmolality

may significantly affect the peripheral circulation The investigation was performed on cats in which the effects of slow intravenous hypertonic infusions on the vascular resistance function in skin skeletal muscle intestine and kidney were analysed before and after regional sympathectomy

Methods

Experiments were performed on 14 cats (mean weight ₋ 7 kg) anesthetized with α-chloralose (50 mg/kg) after induction with ether supplemented with a small dose of pentobarbital sodium (15 mg)

Arterial pulsatile and mean pressures were monitored (via Statham P23 AC transducers) from the right carotid artery and observations were further made of heart rate (Grass Tachograph model 7P4 D) and of blood flows from four different tissues Skin skeletal muscle intestine and kidney After heparinization the regional blood flows were measured with optical drop recorder units inserted in the cognate vein of either tissue other draining vessels being ligated The venous outflows were returned to the animal via a funnel connected to the right and sometimes also the left jugular vein With this technique the regional venous outflow pressures were kept constant at a value of about 5 mm Hg Inflow of blood from the funnel was automatically adjusted so as to keep the extracorporeal blood volume small and constant In the individual experiment blood flows from 2 or 3 regions were measured simultaneously All parameters were recorded on a direct writing oscillograph (Grass polygraph)

The preparations were as follows *Skin* Recordings of blood flow from the left hind paw were obtained by insertion of the flowmeter in the great saphenous vein at the level of the ankle All other draining superficial veins were ligated and drainage through deep veins was prevented by the application of a cuff at the ankle which raised tissue pressure to a level sl ghtly exceeding venous outflow pressure The pads of the paw were carefully ligated to exclude the majority of the arterio venous anastomoses hence observed reactions in the cutaneous circulation can mainly be considered representative of those in nutritive skin resistance vessels *Skeletal mu cle* Blood flow from the right lower hind leg muscles was recorded with the technique described by Kjellmer (1964) *Inte tin* A 15 cm long segment of the jejunum was prepared to permit blood flow recordings according to Folkow *et al* (1963) *K in y* Blo d flow from the left kidney was recorded after cannulation of the ma n renal vein The left o arian or spermatic veins were l gated

The effects of i v iso- and hyperton c infusions on vascular resistance in the 4 regions were stud ed both before and after regional sympathectomy At the end of each experiment the studied tissues we e weighed to permit calculation of blood flow per 100 g tissue Reg onal resistance (arterio-venous pres ure grad ent (blood flow min 100 g tissue)) was determined before (control) and during the infusions and the reactions in the res stance vessels were expressed as per cent change from the control value

Intravenous infusions of isotonic and hypertonic (30 to 50 % solut ons of glucose xylose and sucrose were administered via the right axillary ein usually at a rate of 1 0 ml min Arterial blood mples were intermittently withdrawn from a T tube in the arterial catheter and analysed for plasma o molal ty by thermistor cryoscopy (Advanced Instruments Inc) In 4 expts observations wer made afte intra enous admin stration of propranolol (1 mg kg b wt) Spread of data is gi en below as S E

Results

Hyperosmolality in the arterial blood develops relatively rapidly in muscle exercise and hemorrhage reaching peak levels of 20 25 mOsm/kg H_2O above control within 4 6 min in heavy work (Lundvall *et al* 1972) and in 10 20 min in bleeding (Järhult 1973) In the p esent study the intravenous hypertonic infusion rates were adjusted so as to evoke a similar arterial hyperosmolality in 5 min The experimental protocol was as follows The animal rested for about 30 min after the completion of the surgery After this on isotonic and 2 3 hypertonic infusions of 5 min duration were made permitting full recovery of the circulatory events between the different infusions Samples for arterial plasma osmolality determina ions were withdrawn in the control period and 1 3 5 10 and 20 m n aft the start of the infusion

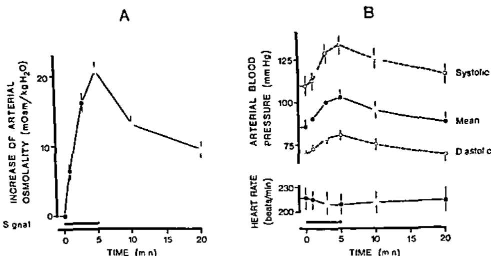


Fig 1 Increase of arterial plasma osmolality above the control level (panel A) and changes of systolic, mean, and diastolic arterial blood pressure and of heart rate (panel B) evoked by intravenous hypertonic infusion (signal) Mean values ± S E. are given for 30 infusion experiments performed in 14 cats

No significant circulatory effects were evoked by the isotonic infusions whereas the hypertonic infusions caused characteristic changes of blood pressure and regional blood flows For a given increase of plasma osmolality the glucose xylose and sucrose solutions evoked responses of quite similar magnitude indicating that the effects were caused by the osmolar change rather than by a specific action of the different solute molecules

Fig 1 panel A shows that arterial osmolality increased by an average of about 20 mOsm/kg H O during the i v hypertonic infusions (n = 30) and then gradually declined in the postinfusion period The concomitantly evoked changes of systolic mean and diastolic arterial blood pressure and of heart rate are shown in panel B At the end of the 5 min infusion period mean pressure had risen by an average of 18 mm Hg and pulse pressure by 13 mm Hg Heart rate was not much affected if anything it tended to decline In the post infusion period there was a gradual recovery of these events

Fig 2 depicts the changes of blood flow and regional vascular resistances (expressed as of control resistance) in skin skeletal muscle intestine and kidney during the hyper tonic infusion (signal) and in the postinfusion period The open circles refer to experiments in which the sympathetic innervation was intact and the closed circles to observations after regional sympathectomy The number of observations is given in the figure The regional blood flows in the control period before infusion are of the order of magnitude noted in larger materials (*e g* Mellander and Johansson 1968) The changes of arterial osmolality caused by the hypertonic infusions were quite similar in the different experim nts depicted in the four panels of Fig 2 the average values being those shown in Fig 1 panel A It can be seen from Fig 2 that hypertonic infusion increased blood flow in all four tissues These effects were to some extent due to the increased perfusion gradient (*cf* Fig 1 panel B) but were mainly caused by decreased vascular resistance as can be seen from the lower part of the diagrams Vascular resistance decreased both in the intact and the

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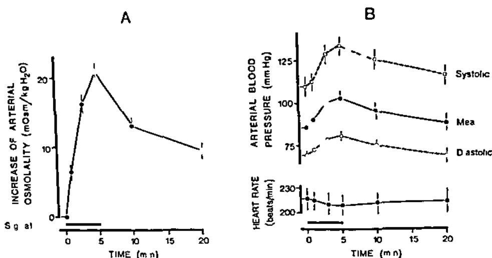


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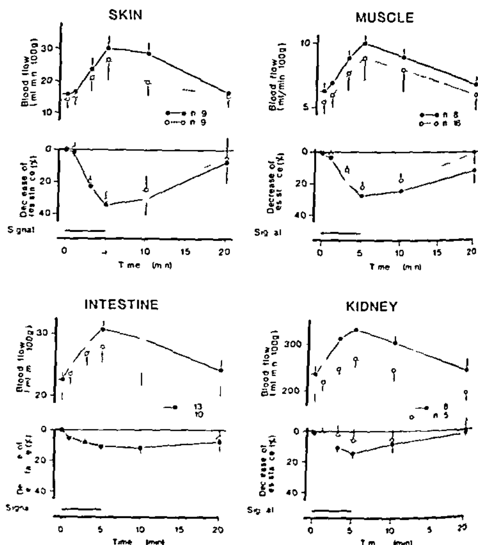


Fig. . Effects of hypertonic infusion (signal) on the regional blood flow and vascular resistance in intact ( ) and sympathectomized (●—●) vascular beds of skin skeletal muscle intestine and kidney Mean values S.E. are given. The concomitant average changes of arterial osmolality and blood pressure are depicted in Fig. 1

sympathectomized regions even if the effects tended to be somewhat less pronounced with intact innervation It follows that the resistance decrease is a local and not a reflex nervous adjustment and it most likely can be ascribed to a direct dilator action of hyperosmolality on the resistance vessels (see below) In relative terms the resistance decrease seemed more pronounced in skin and muscle tissues than in intestine and kidney

The circulatory effects of hypertonic infusion were also analysed after administration of propranolol (4 cats) in a dose (1 mg/kg b.wt.) known to effectively block the beta adrenoceptors Such blockade did not affect the peripheral dilator responses to hypertonic infusion in these experiments but the blood pressure responses was depressed by an

average of 40%. The described dilator effects thus cannot be attributed to possible release of adrenaline from the adrenals

Discussion

The present study has shown that physiological increases of the osmolality of the blood as can occur in exercise or hemorrhage lead to raised arterial mean and pulse pressure (in agreement with Stainsby and Barclay 1971) and to a decrease of vascular resistance in skin skeletal muscle intestine and kidney both before and after regional sympathectomy Blockade of β adrenoceptors attenuated the blood pressure response but did not alter the hypertonic effects on vascular resistance The decreased vascular resistance most likely is due to an active dilator response caused by direct inhibition of vascular smooth muscle tone by hyperosmolality as evidenced by previous in vivo and in vitro experiments (Mellander *et al* 1967 Johansson and Jonsson 1968 Lundvall 1972) Rheological or other passive effects of the hypertonic infusion may be considered small (see Lundvall 1972) but perhaps not entirely negligible due to an osmotic absorption of extravascular fluid and consequent plasma volume expansion (Lundvall *et al* 1972, Atkins *et al* 1973 Jarhult 1973) It may be concluded that moderate (physiological) blood borne hyperosmolality has a significant dilator effect in these four regions of the systemic circulation Hauge and Bø (1971) have previously shown that such hyperosmolality also evokes a clearcut dilatation of the pulmonary resistance vessels

The raised arterial blood pressure in face of dilatation in four hemodynamically important vascular beds is indicative of an augmented cardiac output in turn caused by increased stroke volume since heart rate was not much altered This interpretation is corroborated by previous direct observations of cardiac output and total peripheral resistance by Atkins *et al* (1973) The latter authors attributed the cardiac effects mainly to a direct positive inotropic action of hyperosmolality The present results obtained after interference with β adrenoceptors suggest that hyperosmolality in addition causes an inotropic effect via a moderate excitation of the sympathetic nervous system an opinion also expressed by Wildenthal *et al* 1969 The latter effect may be mediated through postulated p riph ral osmoreceptors (Lasser *et al* 1960) or by an action of hyperosmolality on the central nervous system (Holland *et al* 1960 Mellander and Hillman 1975) The cardiac effects of hyperosmolality to some extent might also have been due to interference with the Frank Starling mechanism in view of the mentioned osmotic plasma volume expansion. An inhibitory reflex adrenergic effect noted in the initial phase during rapid intravenous infusions of strong hypertonic solutions (Raizner *et al* 1973) was not observed in the present experiments with more moderate hyperosmolality

Circulatory adjustment to stress (*e g* exercise or bleeding) is the integrated net result of simultaneous synergistic or antagonistic influences of different control systems on the heart and the peripheral circulation The importance of each control system may be best appreciated by studying its selective effects under standardized experimental conditions The present results taken together with those of Wildenthal *et al* (1969) and Atkins *et al* (1973) suggest that blood borne hyperosmolality is a factor which can contribute to the cardiac adjustments in exercise and hemorrhage at the same time, the hyperosmolality

would tend to oppose the reflex adrenergic constriction of the peripheral resistance vessels in these situations

This study was supported by grants from the Swedish Medical Research Council (B75 14X 10-09A) and from the Faculty of Medicine University of Lund (53482332 3)

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Acta physiol scand 1975 93 135–138

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Relationship between Isometric Endurance and Fibre Types in Human Leg Muscles

By

B HULTÉN A THORSTENSSON B SJÖDIN and J KARLSSON

Received 19 July 1974

Abstract

HULTEN B A THORSTENSSON B SJÖDIN and J KARLSSON *Relationship between isometric endurance and fibre types in human leg muscles* Acta physiol scand 1975 *93* 135–138

Relationship between isometric endurance performance at 50 of maximal voluntary isometric contraction (MVC) and skeletal muscle fibre composition has been elucidated in 19 physical education students This was found to be linear and the equation corresponded to $y = 9\ 35 + 1\ 093x$ $r = 0\ 70$ (endurance time expressed in seconds and fibre composition as percent slow twitch muscle fibres (ST) of the vastus lateralis muscle) As it is assumed from previous studies that similar isometric tensions preferentially recruit fast twitch muscle fibres (FT) and that the muscle at the point of exhaustion exhibits maximal values for lactate accumulation, it is suggested that lactate formed in FT fibres is released and stored in nonrecruited ST fibres The ability to sustain similar isometric tension would then be depending on how large the fraction of ST fibres is that can serve as a lactate recipient for lactate producing FT fibres

Isometric tensions corresponding to 30–50% of maximal leg voluntary isometric contraction (MVC) have been shown to produce maximal values for muscle lactate concentrations at the point of muscular exhaustion (Karlsson and Ollander 1972 Karlsson *et al* 1974) Lower and higher relative tensions respectively were demonstrated to cause submaximal phosphagen depletions as well as submaximal lactate accumulations in spite of muscular exhaustion Repeated exhaustive isometric contractions at tensions corresponding to 30–80 of MVC did not change the metabolic pattern obtained after the first contraction no further accumulation of lactate did occur (Karlsson *et al* 1974) Furthermore it has been demonstrated that at isometric tensions corresponding to approximately 25% of MVC or less only slow twitch fibres (ST) were depleted on their glycogen content as indicated by periodic acid Schiff (PAS) stainings (Gollnick *et al* 1974) At tensions in excess of 25 only the fast twitch fibres (FT) were glycogen depleted The interpretation was that only FT were recruited to maintain high tensions whereas at lower tensions only ST were recruited This conclusion about the recruitment pattern seemed to be in contradiction to results from Molbech *et al* (1973) who investigated isometric endurance in muscles with different contractile characteristics and consequently most likely with different fibre

populations. They showed that isometric endurance time at 50% of MVC was longer for muscles with predominantly slow twitch characteristics than for muscles with predominantly fast twitch properties.

To further investigate how fibre composition is related to isometric endurance, 19 subjects were examined at a tension corresponding to 50% of MVC, and muscle biopsies were obtained for fibre-typing. It was demonstrated that isometric endurance at this intensity was positively correlated to the percentage of slow twitch fibres.

Methods

19 students of physical education participated in the study. For antropological and physiological data see Table I. The experiments were performed in an isometric chair as described by Karlsson and Ollander (197[illegible]) by pressing the feet against an immovable bar whereby activating mainly the thigh muscles. MVC was determined on [illegible] consecutive days and from these data individual tensions corresponding to 50% of MVC were calculated. The endurance tests were performed on the third day. The subjects reported in the laboratory early in the morning after a light breakfast and the experiments were performed without any preceding maximal tests. Muscle biopsies (Bergström 196[illegible]) were obtained from vastus lateralis on one of the first two days and analyzed for fibre types according to Gollnick *et al.* (197[illegible]).

Results and Discussion

Individual MVC ranged 19[illegible]–400 kp (mean 245 kp) and fibre composition 3[illegible]–69% ST (mean 54% ST) for the experimental group (Table I). No relationship was found between individual MVC and fibre type composition. Endurance time at 50% MVC ranged 5[illegible]–94 sec and was related to the individual fibre type composition (Fig. 1), demonstrating increased performance with a higher percentage ST fibres. These findings are in accordance with the conclusions of Molbech *et al.* (1973) that endurance is related to the quality of the muscle with respect to fibre type composition. A previous study suggested that isometric tensions in excess of approximately 25% of MVC are produced by the recruitment of FT fibres (Gollnick *et al.* 1974). However, in the present study maximal tensions could not be related to the percentage FT fibres indicating additional factors of significance for peak tension.

Since blood flow at tensions corresponding to 50% MVC are severely restricted with no or very little oxygen delivery to the contracting fibres (for further discussion see Ekblad 1974), the anaerobic glycolysis will be utilized for ATP resynthesis (Karlsson 19[illegible]). Lactate will then accumulate in the fibres and be released into the extra-cellular fluid and the venous blood. Since venous lactate efflux from contracting muscle already during conditions similar to dynamic exercise has been shown to be limited (Karlsson 197[illegible] and

TABLE I. Mean values [illegible] S.E. for age, weight, height, maximal isometric tension (MVC), and percent slow twitch fibres (ST) of the examined muscle ([illegible] lateralis)

Number of subjects	Age years	Weight kg	Height cm	MVC kp	Fiber composition % ST
n = 19	[illegible] 0.8	4[illegible] 3.7	1[illegible]	[illegible]45 [illegible] 14	54 [illegible] 9

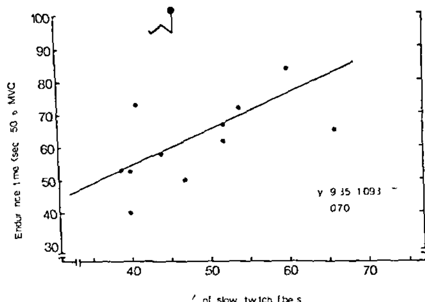


Fig 1 Relationship between ind idual endurance time and percent slow twitch fibres of the examined muscle (vastus lateralis)

Jorfeldt pers com) the rate of lactate accumulation in the contracting muscle during iso metric exercise will most probably be much more pronounced as compared to dynamic exercise The lactate anion in itself has been demonstrated to be a potent inhibitor of lactate formation during *in vitro* conditions (Karlsson Hulten and Sjodin 1974) If this is true also *in situ* it seems reasonable to assume a subsequent arrest of the glycolysis at peak values of lactate accumulation due to the fact that the whole extramitochondrial pool of NAD will be transferred into a reduced form (Sund 1968) If so the release of lactate to other compartments than the venous blood ought to be detrimental for the capability of the muscle to sustain an isometric contraction Nonrecruited ST fibres is one very probable recipient of released lactate In addition ST fibres might be able to metabolize lactate formed in FT fibres The prerequisites for lactate oxidation in skeletal muscle in terms of the heart specific LDH isozymes are present in ST fibres (Karlsson *et al* 1974) Moreover ST fibres have been shown to contain larger amounts of myoglobin than FT fibres (James 1968)

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Acta physiol. scand. 1975. 93. 139–141

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Effect of Substance P on Blood Flow in Canine Adipose Tissue and Skeletal Muscle

By

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In 1931 Euler and Gaddum described smooth muscle stimulating and hypotensive effects of extracts from horse brain and intestine. The active factor was called Substance P (SP). Intravenous administration of purified preparations of SP caused a rapid fall in blood pressure in the rabbit and cat which was not blocked by atropine, antihistamines or ganglionic blocking agents (Pernow 1953 a). The hypotensive effect of SP was presumed to be due to peripheral vasodilation; this was later partially confirmed by studies in man where an increase in skin and muscle blood flow was demonstrated during infusion of SP into the brachial artery (Löfström, Pernow and Wahren 1965).

Recently Chang and Leeman (1970) isolated an undecapeptide from bovine hypothalamus with biological properties indistinguishable from those described earlier for SP which is known to be present in large amounts in the hypothalamus (Pernow 1953 b). This undecapeptide is now available in synthetic form (Tregear *et al.* 1971, Fisher *et al.* 1974) which opens up greater possibilities for pharmacological studies. The present paper reports some actions of synthetic SP on the circulation in skeletal muscle and adipose tissue in the dog.

Methods

7 dogs anesthetized with sodium pentobarbital (30 mg/kg b.wt., with supplement when necessary) were used. Arterial blood gases were analyzed in most animals and if necessary artificial positive pressure breathing was instituted. The subcutaneous adipose tissue in the inguinal region of female dogs was prepared as described earlier (Rosell 1966). Blood flow was measured by means of a drop counter in the main artery. The vein draining the adipose tissue was cannulated with polyethylene tubing and the venous outflow was collected for the analysis of glycerol (Laurell and Tibbling 1966) and free fatty acids (FFA) (Trout *et al.* 1960). FFA and glycerol were also measured in arterial samples in order to be able to calculate net outflow.

In some experiments the adipose tissue was placed in a plethysmograph (Öberg and Rosell 1967) in order to be able to determine the capillary filtration coefficient (CFC). To measure skeletal muscle blood flow the gracilis muscle was isolated from surrounding tissue and blood flow was measured by means of a drop counter in the main artery (Renkin

electrodes Hypocapnia was obtained by an increase of tidal volume hypercapnia by the addition of 6 CO_2 to the inspired gas mixture

The original Oldendorf method was applied in the following manner The right common carotid artery was exposed to permit injection of the test bolus but was allowed to remain patent throughout the experiment Test substances were not injected until Paco had remained constant for at least 20 min A 200 µl mixture of tritiated reference substance and ^{14}C labeled test substance in HEPES buffer was injected rapidly into the right common carotid artery 15 s after injection the animals were decapitated the brain rapidly removed and the right hemisphere used for analysis of labeled substances using a slight modification of Oldendorf's principles

The right hemisphere was disrupted by passing through the nozzle of a narrow gauge syringe The homogenate was divided in two halves each approximately 200 µl in volume and left overnight in a mixture of 1 ml Soluene and 1 ml dioxane at 40 C The following day the solution was bleached for 1 h with 35 hydrogen peroxide at 0 C treated with 450 µl 1 M HCl and completed for β scintillation with 15 ml Insta Gel

Aliquots of the injection mixture were added to non injected brain tissue and prepared as above Blank brain tissue specimens served as background standards and β scintillation took place in a Packard Tri Carb 3003 liquid scintillation spectrometer

Results were calculated from the right hemisphere activity of ^{14}C labeled substance relative to activity of tritiated substance divided by the injectate ^{14}C activity relative to ^{3}H activity This ratio was designated the Brain Uptake Index (BUI) by Oldendorf

4 test substances were used ^{14}C antipyrine ^{14}C-ethanol sodium ^{14}C-D lactate and sodium ^{14}C L lactate reference substances were used ^{3}H water and ^{3}H-ethanol

Tritiated and C labeled compounds were delivered by the New England Nuclear Corporation and the Radiochemical Centre Amersham When carbon labeled ethanol was labeled in the 1 position with a specific activity 5 3 mCi mmol As tritiated substance ethanol was methylene labeled in the same position with a specific activity of 25 mCi mmol Antipyrine was carbon labeled in the N methyl position with a specific activity of 11 mCi mmol The sodium salts of D and L lactic acid were carbon labeled uniformly with specific activities of 9 1 and 40 mCi mmol respectively Tritiated water had a specific activity of 1 mCi g Approximately 0 5 µCi of test substance and µCi of reference substance were used in each injectate bolus

Results

Normal animals

The BUI of antipyrine and ethanol relative to water was studied in normocapnic rats (Table I) In this case antipyrine was mixed with fresh rat serum in the injectate to ensure optimal protein binding of this substance Also shown in Table I are the BUI of D and L lactate *The BUI of antipyrine was lower than that of ethanol Both forms of lactate* seem to be taken up by brain tissue L lactate at a greater rate th... lactate The rate of brain uptake of water is lower ... ethanol but higher than ... rine

Hypo- and hypercapnic rats

In these experiments antipyrin... mixed with HEPES ... r serum The BUI of antipyrine ... apnia declined be protein binding (Table II) Nei... or in hyper-cap significa... of BUI observe...

The ... e of ethanol wa... to water (T signific... BUI was obse... apnia

The h... antipyrine w... e to during no... expected d... rine

TABLE I Brain uptake of lactate antipyrine and ethanol relative to water Normal rats n = number of observations. BUI = Brain Uptake Index ± S E.

n	Test substance	BUI
13	D lactate	8 9 ± 0 9
16	L-lactate	.0 4 ± 1 3
27	Antipyrine	68 4 ± 1 1
19	Ethanol	137 5 ± 3 9

the higher rate of brain uptake of ethanol than of water In hypo- and hyper-capnia how ever the BUI of antipyrine relative to ethanol declined 50 ₒ of the normocapnic value No remarkable change of other variables was observed

Discussion

The Oldendorf method for the study of the unidirectional brain uptake of radiolabeled tracers is well-established One of its main advantages lies in the fact that direct analysis of brain content of test and reference substances is avoided The method is designed so as to make it unnecessary to know the weight of the brain tissue specimens or the volume of the injectate aliquots because the BUI is calculated from relative activities The tissue analysis used in the present study represents only a very minor modification of the original proce dure described by Oldendorf (1970)

The BUI values for D and L-lactate presented in this study agree with results reported by Oldendorf (1971 b) but disagree with results reported by Crone and Sørensen (1970) who found no extraction of D or L-lactate by the brain of dogs These authors used the single injection technique of Crone (1965) of which the Oldendorf method is the reverse Crone s method is based on a non-diffusible reference substance which appears in multiple venous samples after a single arterial injection Conversely the Oldendorf method requires a freely diffusible reference substance which cannot be immediately detected in venous out flow samples

TABLE II Brain uptake of ¹ C antipyrine relative to ³H water at normo- hypo- and hypercapnia All values are expressed ± S E. n = number of observat ons Paco = partial tension of CO in arterial blood BUI = Brain Uptake Index Pa_{O_2} = partial tension of O in arterial blood MABP = mean arterial blood pressure T = rectal temperature

Ant pyrine water		n	Paco mm Hg	BUI of water	Pao mm Hg	apH	MABP mm Hg	T C
Normocapn a	Mean	11	36 9	79 9	135	7 43	160	36 8
	± S E.		0 4	.4	7	0 0	5	0 1
Hypocapnia	Mean	11	24.5	72 4	16	7 51	160	37 0
	± S E.		0 7	1 6	3	0 03	4	0 1
Hypercapn a	Mean	1	64 1	79 5	137	7.4	163	36 9
	± S E.		0 9	0	3	0 01	1	0 1

TABLE III Brain uptake of ^{14}C-ethanol relative to ^{3}H water at normo- hypo- and hypercapnia All values are expressed ± S E. n = number of observations $PaCO_2$ = partial tensi n of CO_2 in arterial blood BUI = Brain Uptake Index. PaO_2 = partial tension of O_2 in arterial blood MABP = mean arterial blood pressure T = rectal temperature

Ethanol water		n	Paco mm Hg	BUI of water	Pao_2 mm Hg	apH	MABP mm Hg	T C
Normocapnia	Mean	8	37 6	1?7 5	1_7	7 39	164	36.7
	± S E.		0 6	3.2	-	0 00_	-	0
Hypocapnia	Mean	10	_3.3	1 0 4	138	7.53	168	36 8
	± S E.		0 4	4 7	5	0 008	4	0
Hypercapnia	Mean	9	61 5	1 7 5	139	7 _1	164	36 8
	± S E.		0 9	_3	7	0 007	-	0 1

Oldendorf suggested (1971 b) that the apparent discrepancy between the extractions of D and L lactate obtained with the two methods was due to an insensitivity to the brain uptake of very small amounts of tracer inherent in Crone s method implying rapid saturation of a hypothetical lactate transport mechanism at higher concentrations of lactate (> 100 μM)

The BUI values of antipyrine and ethanol relative to water at hypo- and hypercapnia indicate little or no effect of changes in cerebral blood flow The study confirms however the marked difference between the uptake rates of the two substances Thus although flow seems to be of no importance within the moderate Pa_{CO_2} range used in the present study the uptake of antipyrine and water is less free than that of ethanol

The uptake rates of antipyrine and ethanol were anticipated by Crone (1965) who noted t the permeability coefficient of ethanol is at least 3 times that of antipyrine across the blood brain barrier Oldendorf (1971 a) found that BUI values for antipyrine water and thanol at normocapnia were 65 100 and 117% relative to water The higher rate of penetration of ethanol was explained by diffusion back into blood but it seems more likely that diffusion limitation is the responsible factor

The findings of the present study have been partially corroborated by Eklöf *et al* (19 4) who studied cerebral blood flow in the rat by a tissue sampling technique with ^{14}C-anti

TABLE IV Brain uptake of ^{14}C antipyrine relative to ^{3}H-ethanol at normo- hypo- and hypercapnia All values are expressed ± S E n = number of observations $PaCO_2$ = partial tension of CO_2 in arterial blood BUI = Brain Uptake Index PaO_2 = partial tension of O_2 in arterial blood MABP = mean arterial blood pressure T = rectal temperature

Antipyrine ethanol		n	Pa_{CO_2} mm Hg	BUI of ethanol	Pa_{O_2} mm Hg	apH	MABP mm Hg	T C
Normocapnia	Mean	10	39 3	51 8	163	7 39	151	36.9
	± S E		0 8	1 8	6	0 01		0
Hypocapnia	Mean	10	8	1 1	164	7 5	160	36 9
	± S E		0 6	0 8	4	0 005	4	0 1
Hypercapnia	Mean	10	63 4	_1 7	1 4	7 19	164	36 5
	± S E.		0 4	1 0	3	0 004	3	0 1

pyrine [1]C-ethanol [3]H water and [133]Xenon comparing the results to blood flow values obtained by [133]Xe-exhalation The results showed that the blood flow values reflect the different extractions of antipyrine water and ethanol and that the differences increased with increased flow implying proper diffusion limitation

In the present study the BUI of antipyrine relative to ethanol displayed a rather curious behavior being 50% below the normocapnic level in hypo- as well as in hypercapnia This behavior was not seen in the experiments with a water reference and is contrary to expectations of true flow dependence suggesting an idiosyncratic interaction between antipyrine and ethanol at abnormal Pa_{CO} levels

We are grateful to Ms Gunilla G dö Ms Bente Mertz and Mrs Elsebeth Steensgaard for expert technical assistance Th study was supported by grants from C C Klestrup s Foundation Copenhagen and the Nord c Insul n Foundation As a medical stud nt, Dr Gjedde held a research scholarsh p from the University of Copenhagen

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Acta physiol. scand. 1975. 93. 150–156

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LDH Isozymes in Skeletal Muscles of Endurance and Strength Trained Athletes

By

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Received 31 May 1974

Abstract

Karlsson, J., B. Sjödin, A. Thorstensson, B. Hultén and K. Frith. *LDH isozymes in skeletal muscles of endurance and strength trained athletes.* Acta physiol. scand. 1975. 93. 150–156.

Muscle biopsy samples were obtained from arm and leg muscles of endurance and strength trained athletes respectively. Total LDH activity as well as occurrence and activity of LDH isozymes were determined. Comparing the results from the athletes with those from non-trained subjects with corresponding fiber compositions, it was found that the endurance athletes had a lower total LDH activity, a higher [illegible] of the most heart-specific isozymes, LDH (1–2), and, on electrophoresis, [illegible] of LDH (4–5) in both arm and leg muscles. As compared to the untrained material the strength [illegible] athletes tended to have a higher total LDH activity, a similar distribution of [illegible] isozymes and, in the leg muscles, a strong cathodal band, corresponding to LDH 5 [illegible] muscle specific isozyme.

Human skeletal muscle has been shown to consist of 2 major fibre types—slow twitch fibres (ST) with alkaline labile myofibrillar ATPase, and fast twitch fibres (FT) with alkaline stable myofibrillar ATPase (Gollnick et al. 1972).

Recently it was demonstrated (Karlsson et al. 1974) that LDH (E.C. 1.1.1.27) activity (V_{max}) increased with increased percentage of fast twitch fibres in humans. Along with the higher LDH activity there was a decrease in the relative contribution of the more heart specific isozymes, LDH 1 and LDH 2, thus indicating that the fast twitch fibres contain a greater concentration of the more skeletal muscle specific LDH isozymes. As the first mentioned [illegible] of athletes, it was of interest to study endurance as well as strength trained subjects according to a similar protocol.

Materials and Methods

Needle muscle biopsies (Bergström 1962) for histochemical classification of muscle fiber types [illegible] et al. [illegible] and LDH isozyme [illegible] were obtained from the [illegible] lateralis [illegible] long distance runners [illegible]

TABLE I Mean values and standard error (S E.) for the percentage of slow twitch fibres in different muscles of *long distance runners cross country skiers and weight lifters*

	Fibre types ST		
	Vastus lateralis	Gastrocnemius	Deltoideus
Weight lifters n=7	49±3	—	57±12
Long distance runners n=7	71±5	60±3	—
Cross country skiers n=13	—	79±4	76±5

(weight lifters and cross country skiers) The athletes were aged 26 years (range 19-34 years) All of them trained and competed regularly although not all of them on the top elite level

The biopsy samples for LDH isozyme stud es were immediately frozen in liquid nitrogen and stored at −80 C until further analyzed After thawing the specimens were sonicated in 0 1 M Tris buffer pH 7 5 The homogenate was immediately analyzed for total LDH activity (*i e* V_m) both in the direction of lactate and pyruvate production using methods based on Lowry and Passoneau (1964) and modified by Karlsson Diamant and Saltin (1968) Homogenates were then stored frozen (below −80 C) until subsequently analyzed for LDH acti ity using different substrates (lactate and ketobutyrate) to distinguish the fraction of the total activity attributable to LDH 1 plus LDH 2 (Rosalki and Wilkinson 1960) Thermoin acti ation at 65 C for 30 min was then applied to distinguish the activity of LDH 1 (Strandjord and Clayson 1961 Wróblewski and Gregory 1961 and Meurman *et al* 1964) The enzyme activity determinations were performed with an LKB 8600 Reaction Rate analyzer (LKB Produkter AB S 161 25 Stockholm Sweden) operating at 340 nm and 37 C (LKB Produkter AB application note MLC/an-4 Smith Brown and Taylor 1970)

The LDH isozymes were also identified by means of qualitative disc-electrophoresis and subsequent *staining as described* by Dietz and Lubrano (1967) For this purpose 50 μl of the homogenate was placed onto columns of 7 5 percent polyacrylamide gel in 5 60 mm tubes The electrophoresis was run for 2 h at 3 mA a tube

A second portion of the biopsy sample was prepared for histochemical analysis Cross sections 10 μm thick were *stained for myofibrillar adenos ne* triphosphatase activity at pH 9 4 after preincubation at pH 10 3 (Padykula and Herman 1955) The fibres in each sample were then identified as slow twitch (ST) or fast tw tch (FT) as described by Gollnick *et al* (1972)

Results

The skeletal muscle fibre distribution patterns in the examined athletes were similar to those reported by Gollnick *et al* (1972) *i e* a high percentage of fast twitch fibres in the strength and a high percentage of slow twitch fibres in the endurance trained muscles respectively (Table I)

In the examined leg muscles of the weight lifters total LDH *activity averaged* 1 44 10 ⁴ and in the endurance trained 0 65 10 ⁴ (long distance runners) and 0 56 10^{-4} mol × g^{-1} min^{-1} (cross country skiers) respectively In relation to fibre type distribution a similar pat tern was observed for total LDH activity as previously described (Karlsson *et a'* 1974) *i e* a lower total LDH activity corresponded to a relatively higher percentage slow twitch fibres (Fig 1) The differences between the weight lifters and the endurance trained athletes were however larger than could be expected from only the difference in fibre composition Compared to an unselected population (Karlsson *et al* 1974) the total LDH activity of the

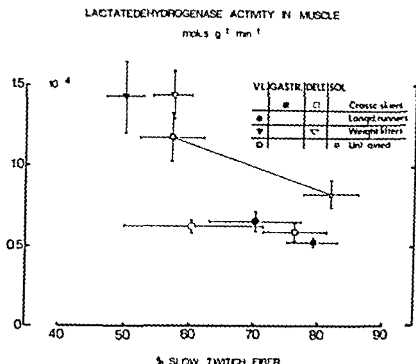


Fig 1 Mean ± S.E. of total lactate dehydrogenase (LDH) activity in skeletal muscles of the different athlete groups in relation to fibre types. The untrained subjects are identical to those presented by C. Ilosk et al 1974 where vastus lateralis and soleus muscles were compared

weight lifters was almost significantly higher and that of the endurance athletes significantly lower for the corresponding fibre type composition (p < 0.05 and p < 0.01 respectively)

The qualitative electrophoretic analysis of LDH isozyme patterns demonstrated bands corresponding to isozymes LDH 1, LDH 2 and LDH 3 in all the muscles investigated from the endurance trained athletes and the deltoid muscles of the weight lifters. The vastus lateralis in the weight lifters was the only muscle that appeared with a distinct band corresponding to the most skeletal muscle specific isozyme LDH 5 (Fig 2). This is in contrast to what was previously found in untrained healthy subjects (Karlsson *et al* 1974) where LDH 5 was always present in skeletal muscle when the percentage slow twitch fibres was in the order of 40–50 percent. Not until the percentage slow twitch fibres approached 90–100 percent did the band corresponding to LDH 5 disappear. This is exemplified with a sample from the soleus muscle (Fig 3) which generally has a high percentage of slow twitch fibres (Gollnick *et al* 1974b)

Although there is a great variation in total LDH activity for a certain fibre composition it has been demonstrated that the activity of certain isozymes, expressed as percent of total LDH activity, is linearly related to the percentage of slow twitch fibres (Karlsson *et al* 1974). To examine whether a shift of the relationship between isozyme activity and fibre distribution was present in endurance and strength trained subjects as indicated by the qualitative electrophoretic analysis (Fig 2) the activity of LDH-1 ... in percent of total



Fig. 2. Electrophoretic separation and staining of LDH isozymes in different muscles of 4 individual athletes (1 is a long distance runner, 2 is a cross country skier, 3 and 4 are weight lifters). The percentages denote the portion of slow twitch (ST) fibres and the figures 1–5 the position of the different LDH isozymes (LDH 1 is the most heart muscle specific and LDH 5 the most skeletal muscle specific isozyme, respectively)

activity was related to fibre type composition (Fig 4). The leg muscle of long distance runners and the arm and leg muscles of the cross country skiers demonstrated a significantly higher relative activity of LDH-(1 + 2) for a certain percentage of slow twitch fibres compared to non-conditioned subjects. The corresponding values for the arm and leg muscles of the weight lifters were similar to those of the untrained group. In absolute terms the activity of LDH-(1 + 2) in the long distance runners averaged approximately 0.39 · 10 (leg muscles), the cross country skiers 0.46 × 10⁻ (arm muscles) and 0.44 · 10^{-4} mol · g⁻ · min⁻¹ (leg muscles), respectively. The corresponding values for arm and leg muscles in the weight lifters were 0.65 · 10^{-4} and 0.63 · 10^{-4} mol · g^{-1} · min respectively.

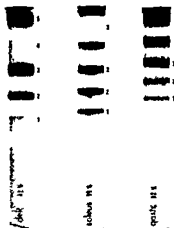


Fig. 3. LDH isozyme patterns in the deltoid, soleus and gastrocnemius muscles of an untrained subject. The percentages denote the portion of slow twitch fibres.

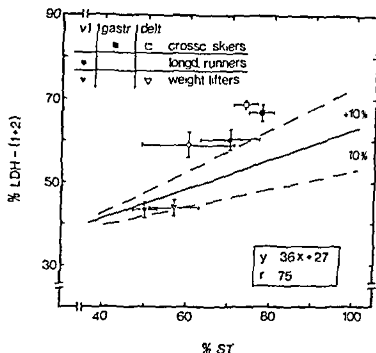


Fig 4 The activity of LDH-(1 + 2) in percent of total LDH activity versus fibre type composition in different muscles of the different athlete groups. The regression line (± 10) represents corresponding values from an untrained material (Karlsson *et al* 1974)

The most heart specific isozyme LDH 1 appeared in a similar pattern as LDH-(1 + 2) *i e* the long distance runners and cross country skiers had higher relative activity of this isozyme The relative contribution of LDH 1 averaged in the long distance runners 40 ± 6 0 (S E) and 45 ± 7 3 (S E) percent in the vastus lateralis and gastrocnemius muscles respectively The deltoid and gastrocnemius muscles of the cross country skiers averaged in this respect 71 2 7 (S E) and 58 ± 3 9 (S E) percent respectively In the weight lifters the contribution from LDH 1 averaged 4 5 ± 3 9 (S E) (vastus lateralis) and 6 3 + 2 0 (S E) (deltoid) percent respectively *i e* a contribution similar to that found in untrained subjects In absolute figures however the activity of LDH 1 was greater in the weight lifters as compared to the normals due to the fact that the weight lifters had a higher total LDH activity

Discussion

Total LDH activity was found to decrease with an increase in percentage of slow twitch fibres thus confirming the results of Karlsson *et al* (1974) However the endurance trained athletes had a lower total LDH activity than an unselected population with the corresponding fibre type composition

Simultaneously the relative activity of LDH-(1 2) in percent of total LDH activity was higher in the endurance trained subjects suggesting that the lower total LDH activity in these athletes might be caused by a decrease in activity of the more skeletal muscle specific

isozymes (LDH 3 4 and 5) This suggestion is supported by the results from the qualitative electrophoretic separations and subsequent staining of LDH isozymes The isozyme band corresponding to LDH 5 normally present in muscles of untrained subjects with the same fibre composition was completely lost in the endurance trained muscles These findings are in agreement with the hypothesis that endurance training shifts the relationship between LDH isozymes to a more heart specific pattern thus affecting and depressing total LDH activity

The rationale for such an hypothesis would be that (i) a linear relationship exists between relative area and percentage of slow twitch fibres (Gollnick *et al* 1972) and that (ii) 5 months of endurance training increased the relative area of the slow twitch fibres (Gollnick *et al* 1973)

If the more heart specific isozymes LDH 1 and LDH 2 are the predominating isozymes in the slow twitch fibres as suggested by Karlsson *et al* (1974) and the relative volume of the slow twitch fibres would increase while the volume of the fast twitch fibres would decrease with endurance training, a similar LDH isozyme pattern as that demonstrated for endurance athletes in this study should be obtained

Total LDH activity in the muscle samples from the weight lifters was higher than for a group of untrained subjects with a corresponding fibre composition The relative activity of LDH isozymes 1 and 2 was not significantly different from that of the untrained The electrophoretic separation of leg muscle samples showed a strong band corresponding to LDH 5 but bands were also present for the more heart specific isozymes *i e* the isozyme picture was similar to the one for nonconditioned subjects As basic knowledge of the effect of strength training on human skeletal muscle fibres at present is scarce as compared to what is known about endurance training it is more difficult to interpret the results obtained for the weight lifters Gollnick *et al* (1972) concluded from their study that strength training was the reason for the enlarged area of the fast twitch fibres in weight lifters This would suggest a corresponding increase in the relative activity of the more skeletal muscle specific isozymes (*e g* LDH 5) in the strength trained muscles This suggestion is not fully supported by the present findings If this is due to an insufficient degree of strength training of the weight lifters or that such an expectation is basically wrong cannot be evaluated at present

In conclusion it is suggested from the present results that the lower total LDH activity in endurance athletes compared to untrained subjects is caused by a relative loss of muscle specific isozymes and a subsequent shift in isozymes to a more heart specific pattern This in turn might be due to a specific effect of the endurance training program on the slow twitch muscle fibres The higher total LDH activity in the strength trained athletes was not accompanied by a comparable shift in isozymes

This study was supported by grants from the Swed sh Medical Research Council (B75-04-4 51-02) and the Research Council of the Swedish Sports Federation

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Acta physiol scand 1975 93 157–166

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The Force–Velocity Relation in Phasic Contractions of Venous Smooth Muscle

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Received 10 June 1974

Abstract

HELLSTRAND P and B JOHANSSON *The force–velocity relation in phasic contractions of venous smooth muscle* Acta physiol scand 1975 93 157–166

The fo ce–velocity relation of the rat portal vein has been studied during regular spontaneous contractions induced by ele ated levels of $[K^+]_o$ and $[Ca^2]$ No satisfactory description of the force–velocity relation was obtained by measuring shortening velocity in afterloaded isotonic contractions Therefore the method of isotonic quick release was used as this permits mechanical studies at specified instants of the contraction–relaxation cycle The temporal de elopment of the force–velocity relation in the phasic response was investigated During a time interval in the rising phase of the contraction at about the time for ma imal dP/dt the force velocity curves were practically identical suggesting a plateau in the intensity of acti e state At later stages of the twitch the curves were progressively d splaced towards the origin the intercepts on both the force and the velocity axis becom ng smaller At the time of the isometric peak the ma imal shorten ng velocity had declined relatively more than the isometric force This is presumably caused by inhomogeneous activation of the muscle at the b ginning of relaxation The maximal forc veloc ty relation in the rising phase of the contraction can be described by Hill s (1938) equat on with the following parameters (at 37 C) $a/P_0 = 0\ 73 \pm 0\ 04$ $b = 0\ 54 \pm 0\ 04$ lengths/s $V_{max} = 0\ 74 \pm 0\ 0$ lengths s (n=7 mean±S E.) The force velocity relation of the portal vein in comparison with other kinds of muscle is discussed

The force-velocity relation of muscle is of central interest for the understanding of the chemomechanical transduction in the contractile apparatus This relation is the quantitative d scription of the muscle s ability to produce power and it is affected therefore by factors which alter the power output notably the length of the contractile element and the intensity of the active state Conversely at a specified length of the contractile element the force-velocity relation at a given instant of time characterizes the mechanical output of the muscle In order to make the several observations necessary to construct just one of the infinite number of force–velocity curves on which the contractile system can operate one need to study the muscle either under a steady state condition for instance during a sustained contracture or at a selected stage of uniform contraction–relaxation cycles (twitches)

In the present study the force–velocity relation of spontaneously contracting longitudinal smooth muscle of the rat portal vein has been determined at different stages of highly uniform contractions which can be induced in the smooth muscle by appropriate alteration of the ionic environment (Johansson 1973) The experiments have been performed by the method of isotonic quick release previously applied to skeletal and heart muscle (e g Jewell and Wilkie 1958 Edman and Nilsson 1968) This technique permits an analysis of the temporal changes in the force–velocity relation during phasic responses which is not possible by merely recording afterloaded contractions as has been done in most earlier studies of the mechanics of smooth muscle A preliminary report of the present study has been presented earlier (Hellstrand and Johansson 1974)

Methods

Rats of the Sprague Dawley strain with body weights between 250 and 400 g were used The rats were killed by cervical fracture and a segment of the portal vein 3–5 mm long was dissected out, carefully cleaned of connective tissue and fat and mounted between metal clamps in the recording apparatus In order to achieve uniform spontaneous activity some of the specimens were trimmed down to about half the circumference of the vessel This produced a more uniform contractile activity with some loss of isometric tension but did not otherwise influence the determinations in any respect

The recording apparatus was essentially as earlier described (Johansson 1973) except that the muscle was now fixed with metal clamps at both its ends One of the clamps was connected to an isometric force transducer and the other to a light lever (equivalent mass 150 mg) connected to a photoelectric displacement transducer The lever was clamped and released by an electromagnet operated stop By means of a spiral spring the lever could be afterloaded in the range 0 to 800 dyn Release could be triggered at preset levels of force P or dP/dt both in the rising and falling phases of the contraction Care was taken to reduce inertial oscillations of the lever by letting it move in a narrow slot containing a drop of oil By suitably choosing the viscosity of the oil inertial oscillations could be effectively cancelled out, whereas the separation between the fast and slow phases of the isotonic response (see Fig 1) was still sharply evident. The output from the apparatus was recorded on a linear direct writing oscillograph (Devices MX 4) and was also stored on magnetic tape (Tandberg Instrumentation recorder series 100) for later replay The preload of the muscle was kept at 50 to 100 dyn

The muscle was first allowed to accommodate for at least 1 h in a physiological salt solution of the following composition in mM NaCl 120 KCl 6 0 $MgCl_2$ 1 2 $CaCl_2$ 2 5 glucose 11 5 and Tris (hydroxy methyl) amino methane (Trizma Base Sigma Chemical Co) 35 The solution was titrated with HCl at 37 C to a pH of 7 4 After the accommodation period the solution was changed to one prepared by replacement of 25 mM of NaCl by an equal amount of KCl and elevation of the content of $CaCl_2$ to 20 mM In some experiments the muscle was left for 1/2 h after the equilibration period in a solution of the same composition as the normal medium except that no Ca + had been added Thereafter the muscle was exposed to a solution containing 25 mM K^+ 20 mM Sr for two minutes before the solution with 5 mM K^+ 0 mM Ca + was introduced This procedure seemed to be still more effective in producing regular uniform contractions in the muscle than the direct transfer to the high K^+ high Ca^{2+} medium No difference in the results of the experiments was detected between the muscles that had been Sr + treated and those that had not All solutions were gassed with 100 O and kept at 37 C. After the experiment the length of the preparation at the actual preload was determined by microscopy with an ocular scale This length was used for expressing the shortening velocity in units of muscle lengths per sec Finally the muscle was cut out from the clamps blotted and weighed on a Cahn electrobalance The force per unit cross sectional area was calculated assuming uniform thickness of the specimen and a density of 1 g/cm³ The tension of the muscle was read directly from the registration

Speed of shortening was determined from the length record by drawing a tangent to the curve For the afterloaded isotonic contractions the speed was determined as the steepest tangent obtained in the isotonic phase For the quick release experiments the tangent was drawn at exactly 100 ms after release Attempts to determine the velocity earlier after the release appeared to give less reproducible results perhaps due to a temporary influence of a damped passive elasticity This effect was independent of the degree of damping of the lever itself and showed a very similar time scale in the different preparations being completed

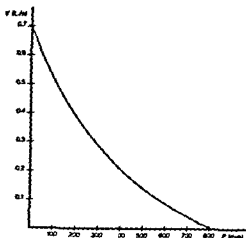
50–75 ms after the release When the shortening velocity was determined at 100 ms after release highly reproducible results were obtained

Results

I *The force–velocity relation as determined by afterloaded contractions and by isotonic quick release*

All the experiments of this study were performed on rat portal vein preparations in which a highly regular phasic activity consisting of contractions with a duration of about 2 s and a frequency of about 6/min had been induced by increasing the external concentrations of K^+ and Ca^{2+} to 25 mM and 20 mM respectively (see Methods) The recordings in the left part of Fig 1 show afterloaded isotonic contractions against two different loads In the right hand recordings the same two afterloads were applied by quick release at a given stage of the isometric tension development It is seen that in contrast to the afterload method the quick release method allows the determination of the shortening velocity for different loads in a unique mechanical state of the muscle since the history of the contraction up to the moment of release is the same for all loads In terms of the Hill model of muscle (Hill 1938) the fast phase of the isotonic recording after release represents the recoil of a series elastic element and the slower phase the shortening of a contractile element alone the series elasticity now having adjusted its length to the force represented by the afterload Therefore if the shortening velocity is determined immediately after release both the time in the contraction cycle and the length of the contractile element should be the same for all determinations Clearly only afterloads less than the instantaneous isometric tension are compatible with shortening of the series elasticity Application of greater loads would involve stretch of the muscle and have not been considered in the present investigation. For reasons discussed in the Methods section it was decided to measure the shortening velocity at 100 ms after the release Thus although the time in the contraction cycle was the same for all determinations with the same point of release the contractile element length would differ slightly when shortening velocities were different In releases against the lightest loads the muscle had shortened by at most 6 of its length before the velocity was determined This small variation should be compared to the large differences in both time of determination and contractile element length occurring when a force–velocity curve is constructed from experiments with afterloaded isotonic contractions Moreover the quick release method allows determination of the force–velocity relation at the peak of the contraction and during the relaxation as well as during the contraction phase

Fig 2 shows force–velocity data obtained for the same muscle by afterloaded contractions (open circles) and by quick release in the rising phase of the contraction (filled circles) The solid line is fitted to the quick release points by Hill s (1938) equation (see further below) The afterload data are seen to deviate towards lower velocities at lighter loads as compared to the quick release data The likely explanation for this is that at light loads the afterloaded isotonic contractions set out very early before the active state has been maximally developed Shortening velocity in the early part of the isotonic response is therefore low because of the low intensity of activation and later it may be inhibited by the rapidly decreasing length The quick releases were all made in the contraction phase at about 3/4 of peak tension at which time the active state seems to be fully developed (Johansson 1973)



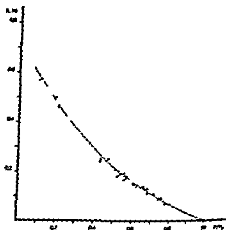


Fig. 5 Force-velocity curves in rising phase of contraction and at isometric peak Same muscle as in Fig. 2. Open circles Release at isometric peak Solid line Maximal force-velocity curve of rising phase from Fig. 2.

Fig. 6. Collected data from seven experiments. Force and velocity values in normalized units Broken line fitted by Hill's equation. $a/P_0 = 0.72$, $b = 0.54$ lengths/s.

III *Characterization of the maximal force-velocity relation*

In Fig. 2 the solid line was fitted to the force-velocity points obtained with releases in the rising phase by use of the equation

$$(P + a)(V + b) = b(P_0 + a) \tag{1}$$

(Hill 1938) Here P is the load on the muscle V is the shortening velocity and a and b are constants with the dimensions of force and velocity respectively P_0 is the force exerted by the contractile element when its length is not changing. The value of P_0 is not in general known during a phasic contraction, except at the peak where it is equal to the isometric tension. P_0 therefore has to be found by extrapolation. This has been done in the present work by means of a procedure based on the equation

$$P = A \exp(V/B) \mp F \tag{2}$$

(Aubert 1956) Here the constants A and F have the dimension of force and the constant B that of velocity The minus sign applies to shortening of the muscle Eqn (2) has been shown to account for the force-velocity relation of frog skeletal muscle equally well as eqn (1) within the experimental error and in the present context it has the advantage of permitting an easy determination of P_0. If the constant F which is thought of as representing an internal load, is chosen suitably a plot of ln (P + F) against V yields a straight line from which P_0 can be found. The value of P_0 obtained in this way is then substituted into a linear form of eqn (1) from which the constants a and b can be found by linear regression analysis.

It was found that a quantitative comparison of the maximal force-velocity curves in

TABLE I Parameters of the force–velocity curves in the rising phase and at the peak of the contraction–relaxation cycle

Exp no	Active tension dyn/cm²	Rising phase					At peak		
		P_0 dyn/cm²	P(t) dyn/cm²	a/P_0	b lengths/s	V_{max} lengths/s	a/P_0	b lengths/s	V_{max} lengths/s
1	3.0×10^5	2.8×10^5	2.1×10^5	0.60	0.41	0.69	0.38	0.17	0.44
2	2.2×10^5	2.3×10^5	1.6×10^5	0.67	0.54	0.80	1.20	0.51	0.43
3	1.6×10^5	1.5×10^5	1.1×10^5	0.92	0.76	0.83	2.27	1.24	0.53
4	1.2×10^5	1.2×10^5	0.7×10^5	0.74	0.50	0.68	0.68	0.31	0.45
5	1.9×10^5	1.9×10^5	1.4×10^5	0.76	0.5[illegible]	0.72	0.92	0.40	0.40
6	1.8×10^5	1.9×10^5	1.5×10^5	0.69	0.48	0.70	0.39	0.[illegible]3	0.59
7	1.7×10^5	1.9×10^5	1.5×10^5	0.76	0.59	0.78	1.77	1.00	0.57
Mean ±S.E.	$(1.9\pm0.2)\times10^5$	$(1.9\pm0.2)\times10^5$		0.73 ±0.04	0.54 ±0.04	0.74 ±0.04	1.1 ±0.3	0.6 ±0.[illegible]	0.49 ±0.03

different experiments is possible if the loads on the muscle are expressed as fractions of P_0 and the velocities in units of muscle lengths per sec. Fig. 6 shows collected data from seven experiments. The interrupted line is fitted to the data according to the procedure outlined above.

In Table I are shown the active tensions exhibited by the muscles and the parameters of the force–velocity curves obtained in the rising phase of the contractions and at the isometric peak respectively. The maximum active tensions found in our study were in the range 1.2 10 dyn/cm² – 3.1 × 10 dyn/cm². These values are relatively low compared to those reported for other smooth and striated muscles. Our experiments were performed at a low passive tension 50–100 dyn in order to minimize influence of parallel elasticity. At this length the muscle is in the rising phase of its length–tension curve and does not develop its maximal active tension. No attempt was made to determine length–tension diagrams for the muscles used in the dynamic experiments since the pattern of regular spontaneous activity is easily disturbed by manipulation of the muscle length.

In the columns of Table I pertaining to the rising phase P_0 is that calculated from eqn (2) and P(t) is the tension at which the releases were made. It is seen that in the rising phase *the values obtained for* a/P_0, b and V_{max} are quite consistent between experiments whereas at the peak there is a considerable spread in the parameters a/P_0 and b. V_{max} generally was lower at the peak than in the rising phase.

Discussion

The Hill equation for the force–velocity relation originally proposed for frog sartorius muscle (Hill 1938) has since been shown to hold for other kinds of skeletal muscle (*e.g.* Close 1972) as well as for cardiac muscle (*e.g.* Edman and Nilsson 1968) and the few kinds of mammalian smooth muscle so far investigated (see below). In the present study it has been shown to account also for the force–velocity relation of the spontaneously active vascular smooth muscle of the rat portal vein. The apparent general applicability of the equation

suggests that the chemomechanical transduction in all kinds of muscle has important properties in common

The equation was classically formulated on the basis of experiments with afterloaded isotonic contractions of tetanically stimulated muscle Essential for this type of study is that the active state reaches its maximal level very rapidly upon stimulation and further that the shortening velocity is not affected by differences in contractile element length occurring in the range over which measurements are made The quick release technique operates on the assumption that each mechanical state of the muscle is characterized by a unique force–velocity relation This is determined by making releases against different afterloads at specified instants of a repeated isometric response The technique is therefore suitable when the above criteria are not fulfilled as is the case with muscles which show a gradual onset of the active state and/or large internal shortening during isometric contraction e g cardiac muscle and smooth muscle Furthermore the quick release technique offers the possibility to study how the contractility of the muscle changes during the course of a phasic response such as a twitch

Inducing regular spontaneous activity in the rat portal vein by alteration of the ionic composition of the bathing medium seemed to be a useful approach to the study of the mechanics of this muscle with maintenance of its phasic character The phasic contractions are not strictly single twitches but are associated with short bursts of action potentials propagating along the preparation with a velocity of about 1 cm/s (see concomitant paper by Johansson and Hellstrand) Although the duration and frequency of the contractions are stable for hours in any single preparation these parameters vary between different muscles As evident from the results of the present investigation however the temporal changes of the instantaneous force–velocity relation are qualitatively similar between experiments. In the rising phase of the contraction at about the time for maximum dP/dt the shortening velocities against given loads appear maximal The velocities are technically easy to determine during this period and well reproducible force–velocity curves are obtained The exact time for the determination is not crucial since the force–velocity relation of the individual muscle remains unaltered for some tenths of a second (cf Fig 4) It seems therefore that in this phase of the contraction the muscle is at a uniformly maximal activity and it appears justified to consider the mechanical properties of the multicellular preparation as representative of the state of affairs also at the level of the individual cells A quantitative comparison between such maximal force–velocity curves from different muscles provides consistent values for the constants of the Hill equation (Fig 6 and Table I)

Later on in the phasic contraction the picture is more confused At the time for the isometric peak tension the value of V_{max} has declined significantly from its maximal level whereas P_0 appears essentially unchanged For the interpretation of these results it should be kept in mind that we are dealing with a multicellular preparation with some asynchrony between cells in their activation–inactivation process (see above) The geometrical distribution of activity should influence the mechanical properties seen in the preparation as a whole In particular if the deactivation process proceeds longitudinally (which seems reasonable) V_{max} should be expected to decline as soon as the level of active state has declined below maximum even in a small proportion of the cells P_0 for the entire muscle

would be less affected since the total cross section of part of the muscle is still maximally active and the partially inactivated cells might be able to carry a load larger than their instantaneous P_0 by being slightly extended The latter mechanism could account for the slow decay in P_0 if the extension of the force–velocity curves to negative shortening velocities would deviate towards the force axis as has been reported for frog sartorius and tortoise retractor penis muscle (Katz 1939) Asynchrony between cells at the time of the isometric peak probably explains the considerable scatter in the parameters of the Hill equation between different experiments (Table I) Such asynchrony may persist at later stages when all cells are relaxing Consequently it is difficult to quantitatively relate the mechanical behaviour of the entire muscle to that of the individual cell The fact that a decrease of both P_0 and V_{max} is observed in the whole muscle during relaxation (Fig. 4) would seem to indicate however that this is a qualitative feature of decreasing activation also at the cellular level In a previous study of K^+ contractures in portal vein we found that variations in P_0, produced by changes in [Ca]$_o$, were associated with insignificant chances in V_{max} (Hellstrand Johansson and Ringberg 1972) However in those experiments the force–velocity curves were obtained at different lengths of the contractile element In that respect a comparison of force velocity curves from the rising and falling portions of the phasic contractions should be more relevant (Fig 4 above)

As indicated above only the maximal force–velocity curve in the rising phase of the contractions deserves quantitative analysis (Table I) The V_{max} attained by the rat portal vein is high compared to values reported for other smooth muscles (*e g* Csapo 1962 Åberg and Axelsson 1964 Stephens Kroeger and Mehta 1969 Gordon and Siegman 1971 Meiss 1971 Herlihy and Murphy 1974) Some of these studies have been carried out on muscles which are intrinsically tonic and in which a low V_{max} is therefore to be expected The phasic smooth muscles which have been examined have been tetanized electrically or brought into contracture by K or drugs Our impression from studies on portal veins in K^+-contracture (Hellstrand *et al* 1972) is that the muscle under these conditions shows a somewhat lower V_{max} than obtained in the present work (*cf* also Hardung and Laszt 1966) The release experiment in the contracture state with a steady tension level before the release is easier to analyze since P is known and the only time-dependent effects occurring are those caused by the release itself On the other hand the K -contracture represents a somewhat artificial state of the muscle in which mechanical or biochemical alterations might affect the force–velocity relation Besides V_{max} *the constants a/P and b found in the present study are* considerably higher than what has been found for other smooth muscles This is reflected in the shape of the force–velocity curve which has a rather weak curvature High values of *a/P and b with slight curvature of the force–velocity curves* were reported for rabbit paillary muscle by Edman and Nilsson (1968) also using quick release experiments with a damped lever In the lack of thermodynamic data for vascular smooth muscle no conclusions can be drawn regarding the significance of the values found for the parameters

The present study was supported by grants from the Swedish Med cal Research Council (8 14X) from the Medical Faculty of the Un ers ty of Lu d and from AB Hässle Göteborg The competent techn cal ass st n e of Mrs. Monica Heidenholm Mrs Monica Lundahl and Mrs Gunh ld N lsson is gratefully acknowledged

The present paper describes differences in the phase of relaxation between isometric and afterloaded isotonic contractions in the smooth muscle of the rat portal vein when regular phasic contractions had been induced by elevated levels of [K+] and [Ca++]. It appears that the changes observed in the course of relaxation in this muscle may be explained without necessarily invoking changes in the time course of the active state.

Methods

Rats of the Sprague Dawley strain with body weights between 250 and 400 g were used. After the animal was killed by cervical fracture the abdomen was opened and the portal vein dissected free from peritoneum and fat. A longitudinal strip of the vein 4 to 8 mm in length and about 1.5 mm in width was prepared. This corresponds to about half the circumference of the vessel between the entrance of the splenic vein and the hepatic hilus.

Electrophysiological experiments designed to elucidate temporal and spatial aspects of the excitatory process in the smooth muscle were performed on the relatively longer strips (7 to 8 mm). The preparation was mounted horizontally in a thermoregulated organ bath filled with physiological salt solution (see below). One end of the strip was fixed to a muscle holder, the other end to a force-displacement transducer (Grass FT 03). A passive force of about 300 dyn was applied. Three glass pipettes with tip diameters of 25 to 100 μm were gently pressed against the tissue for extracellular recording of electrical activity. The potential changes between each of these electrodes and an indifferent electrode consisting of a metal plate at the bottom of the bath were recorded together with the isometric contractile force on a Grass polygraph. Shifts in DC potential were eliminated by 90 μF capacitors in the circuits. The method used for the multiple site electrical recording was thus essentially that described by Ljung and Stage (1970).

Mechanics of portal vein contractions were studied by mounting the strip in an apparatus which allowed simultaneous recording of force and shortening. One end of the preparation was attached to a force transducer and the other end to an isotonic lever which could be clamped or released by an electromagnet system. A preload of 50 to 100 dyn was applied. The afterload could be varied by means of a spiral spring on the axis of the lever. The mechanical apparatus has been described in detail elsewhere (Johansson 1973). The only modification introduced for the present study was that metal clamps were used for fixing the muscle both to force transducer and isotonic lever. Changes of length and force were recorded in the present study by a linear direct writing oscillograph (Devices MX4). The preparations were allowed to accommodate for at least 1 h in a trisbuffered Krebs solution of the following composition in mM: NaCl 1?0, KCl 6.0, $MgCl_2$ 1.?, $CaCl_2$?.5, glucose 11.5 and Tris (hydroxymethyl) aminomethane (Trizma Base, Sigma Chemical Co.) 35. This solution was titrated with HCl to a pH of 7.4 at 37° C. In order to induce a regular spontaneous activity with uniform phasic contractions in the smooth muscle the preparations were transferred to a solution containing ?5 mM K^+ and 20 mM Ca^{++} as described earlier (Johansson 1973). We observed by coincidence in other experiments that regular activity occurred with even greater consistency in this solution if the muscle had previously been exposed to a K^+-high medium containing Sr^{++}. In some of the present experiments a period of exposure to ?5 mM Sr^{++} was therefore inserted before shifting to the medium with ?5 mM K^+ and ?0 mM Ca^{++} as described in the concomitant paper. All experiments were performed at 37° C and the solutions were gassed with 100% O_2.

Results

It was found earlier by sucrose gap experiments (Johansson 1973) that the uniform phasic contractions in the portal vein are related to electrical discharges consisting of several spikes and lasting about 0.5 s. An analysis of the time course of the active state indicated that this increased gradually over the first 400–600 ms. It could not be decided from those experiments whether the gradual increase in the active state was due to graded activation of the individual cells or to slow spread of excitation in the tissue. The present experiments with

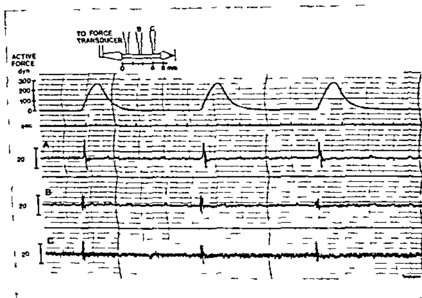


Fig 1 Electrical and mechanical recordings from portal vein Top record isometric force Records A B and C electrical AC registrations from extracellular electrodes A B and C located as shown in inset Electrical activity is propagated along the muscle at about 1 cm/s Excitation is virtually complete in the early rising phase of isometric contraction

both electrical and mechanical recording were performed in order to provide further information on the temporal and spatial aspects of excitation during the regular phasic activity

Results from one of the electrophysiological experiments are illustrated in Fig 1 The uniform phasic contractions were triggered by spontaneous electrical discharges which could be registered from all three recording sites located along the strip as indicated in the figure It appeared that in this preparation excitation started close to electrode C and spread over B to A at a rate of about 1 cm/s The configuration of the electrical signals as detected with this technique was quite variable between experiments and also within individual experiments (*cf* Ljung and Stage 1970) The results indicated that the electrical excitatory process was completed throughout the preparation at a time when isometric force had reached only about half its maximum this was true even in cases where excitation appeared to emerge from one end of the strip as in Fig 1 Also the earlier sucrose gap study indicated that the beginning of the fall in dP/dt approximately coincided with the repolarization This temporal relation between the electrical and contractile events is of some importance for the discussion of the following mechanical experiments

Fig 2 illustrates the differences in the course of relaxation between isometric and isotonic contractions observed in portal vein preparations which maintained uniform mechanical activity In this figure reproduced tracings from 4 different contractions have been superimposed Curve *d* shows the purely isometric response obtained when the lever was clamped through the entire contraction–relaxation cycle With the lever released the contractile force increased to the afterload level of 350 dyn after which isotonic shortening occurred

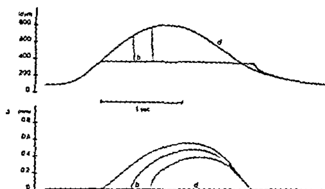


Fig. 2. Superimposed records from isotonic and isometric contractions of portal vein Upper records force Lower records shortening. *a* Isotonic contraction starting when isometric force has reached the afterload of 350 dyn *b* and *c* Muscle released to afterload at later stages of isometric contraction *d* Control isometric response

(*a*) A comparison of the relaxation phases in these two responses shows that the muscle under isotonic relaxation (*a*) was able to carry the afterload for a longer period than one would expect from the isometric curve (*d*) Force dropped below 350 dyn in the isotonic response about 125 ms later than in the isometric one However once the isotonically relaxing muscle has returned to the original length it shows a rapid fall in force so that it quickly approaches the isometric relaxation curve In the final part of the response the two curves coincide Curves *b* and *c* in Fig. 2 show that the above difference in time course between isotonic and purely isometric relaxation appeared also when the muscle was released to the afterload level at later stages of the isometric contraction phase

It was of interest to see how the temporal difference in the isometric and isotonic relaxation depends on the magnitude of the afterload Fig 3 illustrates results from 1 expt in which several different levels of afterload were employed The figure shows superimposed recordings of the purely isometric and of 5 afterloaded isotonic contractions It can be seen that in all cases the plateau of the force curve in the isotonic responses lasts beyond the time at which the isometric curve has dropped to the respective levels of force but the difference in time is largest for the intermediate afterloads and decreases for higher and lower loads After the plateau the force in the afterloaded contractions falls rapidly so that the curves invariably join the terminal part of the isometric curve The broken line in Fig. 3 shows the shift in time for the peak of the length change in the different isotonic responses This shift too is greatest at the intermediate afterloads

Discussion

An inhibitory effect of the shortening process on maintenance or generation of force has been put forward to explain certain features of the course of relaxation in skeletal and cardiac muscle (Jewell and Wilkie 1960 Brady 1965 Edman and Kiessling 1971 Edman and Nilsson 1971 Kaufmann Bayer and Harnasch 1972) Evidence for such inhibition

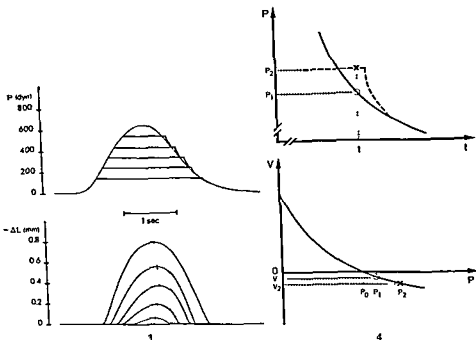


Fig 3 Superimposed records from isometric contraction and from afterloaded isotonic contractions at 5 different loads Upper records force Lower records shortening Broken line connects peaks of isoton c length records

Fig 4 Upper part illustrates the differences in time course of force registration between isometric (full l ne) and isotonic (broken l ne) relaxation (cf Fig 2) Lower part illustrates hypothetical instantaneous force–velocity curve at time t It is suggested that isotonic and isometric relaxation at t are represented by different points (and O) on this curve

has not been found in the present study of relaxation in smooth muscle of the portal vein By contrast isotonic shortening in this tissue was associated with a temporarily improved ability to maintain force during relaxation as indicated by the prolonged duration of the afterload plateau illustrated in Fig 2 and 3 The terminal fall in force of the afterloaded contraction followed the same course as the control isometric response

Several mechanisms may be discussed as possible explanations for the difference in the course of relaxation between the isometric and isotonic responses in portal vein First an influence of the mechanical conditions on spike generation might be considered in view of the well known effect of stretch on membrane activity in many smooth muscles However a difference in the process of excitation between isometric and isotonic contractions can evidently be excluded as a cause of the prolonged plateau in the latter response From the results illustrated in Fig 1 it seems likely that spike activity had already ceased in responses *a* and *d* of Fig 2 before they deviated mechanically from one another this would definitely be true in the case of *b* and *c* compared to *d* in Fig 2.

An inhibitory effect of isotonic shortening on the elimination of activator calcium appeared as a plausible explanation We considered that the increase of the cell diameter dur

ing shortening might simply increase the average diffusion distances to the superficially located sarcoplasmic reticulum probably responsible for Ca++ uptake (Devine Somlyo and Somlyo 1972) so that relaxation would be delayed However had this been the major mechanism the difference in time course between isometric and isotonic relaxations should have been greatest at the lowest afterloads and should have diminished with increasing loads. This was not the case as shown by Fig 3

If the series elastic element were not strictly elastic but showed some hysteresis this could cause relaxation after an afterloaded isotonic response to deviate from the isometric curve Since the series-coupled element is subject to greater forces in an isometric than in an isotonic contraction with a low afterload it would make a wider hysteresis loop in the former than in the latter case This in turn would imply that when the muscle returns to its original length after isotonic relaxation the contractile element would be longer than at the corresponding stage of isometric relaxation At the muscle lengths used in the present study this increase in contractile element length would be expected to enhance active force Such an explanation cannot be excluded by the present experiments but would probably require that the difference between the isometric and isotonic responses be largest for the lowest afterloads Also the identical terminal portions in isometric and afterloaded responses (e g d and a of Fig 2) would not be explained by hysteresis in the series coupled element

It seems important to point out that differences in the time course of the active state or complex alternatives to Hill s (1938) two-component muscle model must not necessarily be invoked to explain the observed differences in the course of relaxation (Figs 2 and 3) The force–velocity relation of muscle implies that the external manifestations of contraction in terms of force and shortening can vary widely for any given level of active state In order to see how this fact applies to the observed differences between the isometric and the isotonic responses it is useful to conceive of muscle contraction as an event which involves several dimensions namely length force velocity and time We may consider that the output from the chemomechanical energy transduction in the contractile element can always be described by an instantaneous force–velocity curve The muscle can operate on an infinite number of such curves depending on the length of the contractile element and on the degree of activation as determined by the level of intracellular [Ca] In order to describe the time course of the chemomechanical transduction (the active state) during a twitch without having to present a set of complete force–velocity curves it has been customary to use Hill s definition of the active state as the force which the contractile element can bear without changing its length (Hill 1949) The time course of the active state is thus given by the P values of all the instantaneous force–velocity curves on which the muscle operates during the contraction relaxation cycle The pathway taken by the contractile element in the length force and velocity dimensions during this cycle will be determined by the load and by the temporal variations in [Ca+]i

If we compare 2 contraction–relaxation cycles which are performed under different mechanical conditions we may find as in Fig 2 that the relaxing muscle does not return to a given length-force situation at corresponding times in the two cases In order to quantitatively predict such time differences it would be necessary to know exactly the time course of the active state and the dependence of the force–velocity relation on length and level of

activation. Such complete information is not available but the mere existence of a time difference in the direction observed in the present study does not necessarily imply differences in the time course of activation as determined by the temporal variations in $[Ca^{2+}]_i$.

Fig. 4 is intended to show how these arguments may be applied to the isometric and isotonic relaxation of the portal vein. During relaxation the contractile element lengthens under the force of the external afterload (isotonic case) or of the series elastic element (isometric case). To illustrate this situation the force–velocity curves must be extended to negative shortening velocities (lengthening). The upper part of Fig. 4 illustrates the above observation that for a period during relaxation, here exemplified by the time t_1, force is higher (P_2) in the isotonic cycle than in the isometric one (P_1). The lower part of Fig. 4 is suggested to represent the instantaneous force–velocity curve which characterizes the chemomechanical transduction at t_1 as determined by the intracellular $[Ca^{2+}]$ prevailing at this point in time. We suggest that this curve may be identical, or almost identical, in the isometric and the isotonic response (if anything the difference in contractile element length at t_1 would give the force–velocity curve a somewhat higher P_0 value in the isometric than in the isotonic case). Due to the differences in the history of the muscle, *i.e.* the different pathways taken through the force–length–velocity space, the contractile element reaches different points on the instantaneous force–velocity curve at t_1 as indicated by the symbols ○ and ×. As $P_2 > P_1$ also the lengthening velocities are different so that numerically $V_2 > V_1$.

The description in Fig. 4 obviously rests on the assumption that the force–velocity relation can be extended to negative shortening velocities. Results obtained on striated muscles have indicated that this may be done even though the curve does not seem to continue along the hyperbola but deviates towards the force axis (Katz 1939, Aubert 1956). Moreover there appears to exist a limiting load ($> P_0$) above which the contractile state breaks down and the muscle lengthens rapidly ("give"). The present results may then suggest that the vascular smooth muscle remains on the reversible extension of the force–velocity curves throughout the period of relaxation. In striated muscles the effective elimination of activator Ca might cause the P_0 values to fall so rapidly that the contractile element can run into a state of "give" leading to high rates of lengthening. It has been suggested that "give" occurs in frog skeletal muscle during isotonic lengthening which would explain the rapid relaxation observed in this muscle (Katz 1939, Hill 1949). A similar mechanism might be considered for heart muscle. If isotonic relaxation leads to an earlier return of the muscle to a given length–force situation than isometric relaxation this would seem compatible only with a mechanically induced depression of the contractile state or a difference in the time course of $[Ca^{2+}]_i$. It is interesting that the rapid isotonic relaxation of cardiac muscle in the study of Kaufmann *et al.* (1972) was abolished by high $[Ca]_o$ which might reduce the rate of fall of P_0, thereby perhaps preventing the muscle from reaching the "give". Also the high [Ca] of the present study may have been important in this way.

It appears that further information on the force–velocity relation at loads greater than P_0 would be most valuable for the understanding of relaxation in muscle.

Note added in proof After submitting this manuscript we have noticed a paper by A. S Bahler (Amer J Physiol 1971 220 1983–1990) where he examines a difference in the course of relaxation in frog skeletal muscle resembling the phenomenon we have now studied in vascular smooth muscle

The present study was supported by grants from the Swedish Medical Research Council (28 14X) from the Medical Faculty of the University of Lund and from AB Hässle Göteborg The competent technical assistance of Mrs Monica Heidenholm Mrs Monica Lundahl and Mrs Gunhild Nilsson is gratefully acknowledged

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Acta physiol scand 1975 93 175–178

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Carbon Monoxide as a Degradation Product of Cytochrome C and of Ascorbic Acid

By

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Received 18 June 1974

Abstract

WESTMAN G *Carbon monoxide as a degradation product of cytochrome C and of ascorbic acid* Acta physiol scand 1975 *93* 175–178

Cytochrome *c* has been incubated with 4 different reducing agents at 37 C for 4 h at pH 7 0 and the spectral changes and the production of carbon monoxide have been followed Only with hydrazine hydrate cytochrome *c* produced some carbon monoxide Accidentally carbon monoxide formation from ascorbic acid was found amounting to about 0 1 μmol from 15 μmol of ascorb c acid in 24 h under the above conditions

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The heme group of hemoglobin myoglobin catalase and plant peroxidase is degraded to bile pigments in an aerated solution of ascorbic acid or some other reductants (Sjostrand 1949 Sjostrand 1952 Kench 1954 Omura and Sato 1964) Initially the light absorption decreases in the range 500–600 nm and increases above 600 nm but then it decrease generally during these changes carbon monoxide is evolved (Sjostrand 1952) Cytochrome *c* however resists coupled oxidation with ascorbic acid (Kench 1954)

In the present experiments the spectral changes of cytochrome *c* and the evolution of carbon monoxide from this hemoprotein in the presence of some reductants have been followed Carbon monoxide has been identified as a degradation product of ascorbic acid Some experiments on hemoglobin are given for comparison

Materials and Methods

Th oacetic acid 99 100 % hydrazine hydrate 98–100 Fluka purum grade Thioglycoll c ac d 80 Merck für Bakteriologie L(+)-ascorbic acid Merck p a .00 mM sodium phosph te buffer pH 7 0 and 37 C and de o ized water were used in all e periments. No trace of CO f om the water could be detected by either of the methods described below Lyophilized cytochrome *c* was purchased f om DDH Chemicals Ltd. O erseas Di ision Poole England (A_{550} $_{ed}$/A_{280} = 1 1) or from S gma (grade V) Spectral analyses were done on a Beckman DK A

Note added in proof After submitting this manuscript we have noticed a paper by A S Bahler (Amer J Physiol 1971 220 1983–1990) where he examines a difference in the course of relaxation in frog skeletal muscle resembling the phenomenon we have now studied in vascular smooth muscle

The present study was supported by grants from the Swedish Medical Research Council (28 14X) from the Medical Faculty of the University of Lund and from AB Hässle Göteborg The competent technical assistance of Mrs Monica Heidenholm Mrs Monica Lundahl and Mrs Gunhild Nilsson is gratefully acknowledged

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Acta physiol scand 1975 93 179-183
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The Heat Production of Pancreatic β Cells Stimulated by Glucose

By

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Received 1 July 1974

Abstract

Gylfe E and B Hellman *The heat production of pancreatic β-cells stimulated by glucose* Acta physiol scand 1975 *93* 179-183

A recently developed batch microcalorimeter was used for studying heat production in β-cell rich pancreatic islets isolated from obese hyperglycemic mice In the absence of glucose the rate of heat production was 50 nW/islet increasing to 90 nW/islet when the islets were exposed to 20 mM glucose The data obtained are consistent with an increase in the caloric value of oxygen with glucose concentration as might be expected when the β-cells utilize proportionally more carbohydrate as a source of energy

The pancreatic β-cells respond to increased supply of various nutrients with stimulation of insulin release Present knowledge of how the overall metabolism of the β-cells is related to their secretory activity is based on studies of oxygen consumption of pancreatic islets either performed with the Cartesian diver technique (Hellerstrom 1967 Hellerstrom and Gunnarson 1970 Hedeskov *et al* 1972) or micropolarography (Atkins and Matty 1970) This paper describes how the development of a highly sensitive batch microcalorimeter (Wadso 1968) made it possible to obtain complementary data on the general metabolism by recording the heat production in pancreatic islets from obese hyperglycemic mice

Materials and Methods

Chem al Sigma Chemical Co., St. Louis Mo U.S A suppl ed bovine serum albumin (fract on V) N 2 hydroxyethylp perazine N -ethane sulphonic acid (HEPES) and antimycin A Gentamycin and Tylos n were obta ned from Bio-Cult laboratories Ltd Paisley Scotland Crude collagenase (14. U/mg) was supplied by Worthington B ochem cal Corp Freehold N.J U.S A All other chemicals were commercially available and of analytical grade D stilled and deionized water was used throughout the e pts

A imal and isolat o of isl ts Non inbred adult obese hyperglycemic mice (gene symbol *ob/ob*) were taken from a local colony (Hellman 1965) The animals were fasted overnight before being killed by decap tation under ether anesthesia Islets were isolated by collagenase treatment of the pancreas (Lernmark 1974) employing albumin-containing (1 mg/ml) Krebs-Ringer b carbonate solution buffered w th ?0 mM HEPES as basal medium. In each expt. 00-400 islets were isolated from two pancreases and thoroughly washed

Weighing of islets The rates of heat production were preliminarily expressed per islet due to the difficulties encountered in collecting the islets from the microcalorimeter after the expt A rough estimate of heat production per weight was made by recording the mean dry weight of the islets isolated by collagenase in 4 separate expts In each of these expts 50–300 pooled islets were freeze-dried (−40 C 0 1 Pa) over night and weighed on an electrobalance (Cahn Division Paramount Calif U S A)

Instrumentation A batch microcalorimeter (LKB 10700-2 LKB Produkter AB Bromma Sweden) of the construction described by Wadsö (1968) was used in combination with a Keithley 150 B microvolt ammeter and a potentiometric recorder The instrument produces a thermogram i e a recording with time of the difference in heat production rates between two gold reaction cells Each reaction cell consisted of 2 compartments one 2 and the other 4 ml in volume Calibration of the reaction cells was accomplished by feeding a 50 ohm resistor in each reaction cell with a current of 1 mA The microcalorimeter was found to be very sensitive to variations of environmental humidity and was therefore placed in a room with a relative humidity of 25 at 22 C The instrument was also connected to an external thermostatic bath set slightly below the operation temperature (37 C)

Experimental design 4 ml of oxygenated Krebs-Ringer HEPES medium was added to the large compartment and 2 ml medium supplemented with 60 mM D glucose to the small compartment of each reaction cell The microcalorimeter was then allowed to equilibrate overnight Tylosin (10 μg/ml) or Gentamycin (100 μg/ml) were added to the medium to inhibit bacterial growth After equilibration it was checked that the instrument gave a stable base line for 2 h when used for measurements in the 3 μV range After adding 200–400 islets to the large compartment of one of the reaction cells the instrument was allowed to equilibrate for 40–60 min The difference between the base line and the new level attained was equivalent to the rate of heat production from the pancreatic islets in the absence of glucose The heat production was followed for 20 min in the glucose free medium The contents of the two compartments of each reaction cell were then mixed resulting in a glucose concentration of 20 mM The mixing procedure lasted about 1 min and was accompanied by peaks on the thermograms whether islets were present or not. The use of one of the reaction cells as a reference balanced off most of the heat evolved during mixing by friction and dilution The rate of islet heat production at the high glucose concentration was followed for 60 min The sensitivity of the instrument was then decreased to 10 μV and the reaction cell containing the islets was calibrated This calibration made it possible to express the rate of heat production as nW/islet. It was checked whether heat production was due to metabolism by exposing the islets to 10 μM antimycin A for 60 min at 37 C (Edwards *et al* 1972) before they were transferred to the microcalorimeter

Results

The rate of heat production in the absence of glucose was equivalent to about 50 nW/islet The mean dry weight (±S E) of the islets was estimated to 3 9±0 6 μg resulting in a basal rate of heat production of about 13 nW/μg islet dry weight

After raising the glucose concentration to 20 mM the rate of heat production increased to about 90 nW/islet (23 nW/μg islet dry weight) (Fig 1) The heat production then remained elevated at the 80–90 nW level

When islet metabolism had been arrested by pretreatment with antimycin A no heat was produced either before or after adding glucose

Discussion

The recent demonstration of heat generation during leucocyte phagocytosis (Levin 1973 a) suggests that microcalorimetry might be a useful tool also for studying the reversal of this membrane phenomenon ejection of secretory granules by emiocytosis The principal aim of this investigation was to determine whether microcalorimetry at the present state of development could contribute to the understanding of the process of insulin secretion from

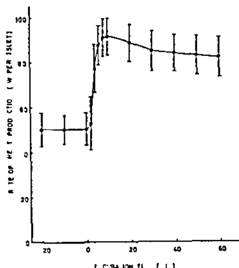


Fig. 1 Thermogram of pancreatic islets isolated from obese hyperglycemic mice After incubation in medium devoid of glucose this compound was added at zero time to a final concentration of 20 mM The rate of heat production was expressed as nW per islet and refers to the mean values ±S E. for 5 separate experiments

pancreatic β-cells The studies were performed with pancreatic islets isolated from obese hyperglycemic mice as representing a fairly pure population of the insulin producing β-cells (Hellman 1965) Glucose was used as a test substance because it stimulates the metabolism (Hellman *et al* 1974) and the release of insulin (Hellman *et al* 1974 Lernmark 1971) from these islets in a well-defined manner

According to factory specifications the LKB 10700-2 batch microcalorimeter has a sensitivity of 1 μW In the present investigation the most sensitive instrumental range had to be used to measure rates of heat production in the order of 5–30 μW At this high sensitivity heat equilibration after cleaning the reaction cells required a long time Occasionally steady state levels were not reached even after 18 h Another technical problem arose from the base line shifts often occurring after the mixing of the 2 compartments of the reaction cells This shift studied in experiments performed in the absence of islets was in most cases small but sometimes equivalent to a change in the rate of heat production of as much as 2–3 μW Mixing of the cell compartments was in itself associated with heat production by friction and dilution of the glucose solution The latter processes were mainly balanced off by the reference cell and these types of heat evolutions only affected the thermograms during the first 8 min after mixing

When expressed per μg dry weight the rate of heat production by the islets was about 13 nW in a glucose free medium and about 23 nW in the presence of 20 mM glucose In starved obese hyperglycemic mice the dry weight (mean ±S E) of the individual β-cells has been determined by micro-interferometry to be 183 ±4 pg (Petersson 1968) Using these figures the rate of heat production can be estimated as about 2 4 pW/β-cell in the absence of glucose and 4 2 pW/β-cell when 20 mM glucose is present Such rates are within the same order as found in leucocytes where heat production has been reported to be about 5 9 pW/cell (Levin 1973 b)

It can be inferred from current data about the islet production of lactate (Hellerstrom

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Acta physiol scand 1975 93 184-194

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Influence of Peritubular Hydrostatic and Oncotic Pressures on Fluid Reabsorption in Proximal Tubules of the Rat Kidney

By

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Received 1 July 1974

Abstract

ÅGERUP B *Influence of peritubular hydrostatic and oncotic pressures on fluid reabsorption in proximal tubules of the rat kidney* Acta physiol scand 1975 93 184-194

Proximal tubular reabsorption was measured with the split droplet technique under the following conditions Group 1 Star vessel perfusions with ultrafiltrate in the presence of human serum albumin (HSA) and with ultrafiltrate alone Group 2 Capillary perfusions under locally high flow rates with ultrafiltrate in the absence or presence of HSA Group 3 Subcapsular perfusions with ultrafiltrate of high HSA concentrations but under low hydrostatic pressure and ultrafiltrate of low HSA concentrations but under elevated hydrostatic pressure In Group 1 the presence of HSA increased the reabsorptive rate whereas ultrafiltrate alone had no provable effect on the reabsorptive rate In Group 2 a flow dependent decrease of the reabsorptive rate that was not influenced by the presence of HSA was observed Finally in Group 3 high concentrations of HSA did not alter the reabsorptive rate whereas elevation of the hydrostatic pressure decreased the reabsorptive rate The results cannot be explained on the basis of a simple passive mode of action of colloid osmotic and hydrostatic pressure on the reabsorptive rate The possibility of a direct tubulae capillary transport route is discussed

It is assumed that the reabsorption of fluid from the proximal tubule in the capillary is dependent upon active as well as passive transport Active transports are located in the tubular cells and constitutes the main basis for fluid to be transported through the cells In addition it has been suggested that some reabsorption of fluid is caused by wholly passive forces (Persson Ågerup and Schnermann 1971) From a morphological point of view after passage through the cells the fluid has to be transported over the basement membrane through the interstitium and across the capillary wall These transports are driven by forces like hydrostatic and oncotic pressures

A number of reports indicate that the absolute tubular reabsorption is related to the postglomerular capillary protein concentration (or rather oncotic pressure) (Vereerstraten and Toussaint 1968 Spitzer and Windhager 1970 Deen Robertsson and Brenner 1973) Furthermore estimates of interstitial (*i e* subcapsular) hydrostatic and oncotic pressures support the idea of a passive transport of fluid through the capillary wall as well as through the basement membrane (Wunderlich *et al* 1971 Ott Navar and Guyton 1971 and Wolgast

et al 1973) Another indication for the role of the interstitial space mediating proximal re absorption is the notation reported by Wolgast *et al* 1973 that the pressure difference over the tubular wall was decreased under extracellular volume expansion a condition known to result in reduced reabsorption Thus far however no direct experimental evidence has been put forward that shows the relation between interstitial physical forces and tubular reabsorption on the intact kidney Imai and Kokko (1972) as well as Grantham Qualizza and Welling (1972) in contrast to Horster *et al* (1973) have reported that an increased protein-concentration outside an isolated rabbit proximal tubule enhances the reabsorp tion of fluid

The purpose of the present study was to evaluate the functional significance of the renal peritubular (interstitial) physical forces with respect to proximal tubular reabsorption The peritubular capillaries as well as the interstitium were subject to changes in oncotic and hydrostatic pressures and at the same time the reabsorptive rate was measured with the split-droplet technique The reabsorption increased when the peritubular capillaries were perfused with hyperoncotic solutions whereas these solutions had no provable effect when perfused through the interstitium A marked depression of reabsorptive rate was noted when the hydrostatic pressure was increased in the capillaries This depression however was unaffected by decapsulation and the presence of colloids When increasing the inter stitial hydrostatic pressure the depression of reabsorptive rate was modest The results may be interpreted as indicative of a direct transport route for the bulk of the reabsorbed fluid from the tubule to the capillary

Methods

The experiments were carried out on white male rats of the Sprague Dawley strain weighing 180–300 g. The animals were fed on a regular pellet diet and allowed free access to water Anesthesia was induced by an intraperitoneal i jection of Inactin® (110 mg/kg b wt) After trach ostomy the animal was put on its right side on a servoregulated heating pad The right jugular v in was cannulated and used for a constant infus on of saline at a rate of 3 ml/kg and hour The left kidney was exposed via a flank incision After be ng placed in a lucite cup the k dney was f xed in pos t on by pouring super-cooled (37 C) agar solution (2 agar gar in saline) around it. A few drops of m neral oil covered the upper most e posed part of the kidney throughout the experiment The experiment was started about 90 min after induction of the anes thesia *Net reab pti n* of tubul r fluid was measured accord ng to Gertz (1963) with the following mo-difications Sudan black stained castor o l was injected w th a single barreled cannula (marked a in Fig 1) proximal to a straight portion of a proximal tubule The droplet was formed by allowing ultrafiltrate to pass the puncture hole It was then isolated from the luminal fluid by injecting more oil By asp rating f ltered fluid suff c ent to keep the droplet in pos tion and at the same time allowing the proximal oilblock to dr ft d stally the intradroplet hydrostatic p essure could be kept fairly stable

Sequenced photographs e ery 5 second were taken using a Cannon F1 camera with a motordr e unit includ ng a timer and a flash Black and white photo raphs were further enlarged on paper to a f nal magnificat on of about 300 times The evaluat on of the photographs was made by a person to whom the experimental maneu ers were unknown

M c op f lon sy tem A 50 μl Hamilton syringe mounted on a Sage pump was connected to a p pette (outside diameter 6 μm) with a th ckwalled polyethylene tube The syringe as well as the tube was filled with mineral oil A perfus ng condition was considered acceptable if the flow from the cannula stopped with n 10 s after the pump was stopped.

Perf io s l t o Ultraf ltrate from rat plasma was used directly after adjusting pH to 7 5 by add ng a few drops of 0 01 M HCl. The album n solut ons were daily made by add ng free e dr ed human serum albumin ((HSA) Kabi Stockholm Sweden) to the ul rafiltrate

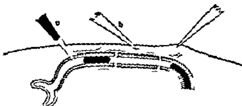


Fig. 1 Schematic drawing illustrating the relative positions of the cannulae used for (a) measurement of reabsorptive rate (b) perfusion of the capillaries and (c) perfusion of the subcapsular space

Star vessel perfusions The perfusion pipette was inserted into the star formed branching of an efferent arteriole that supported a straight superficial proximal tubule in such a way that the perfusate was uniformly directed in the respective branches It is likely that the perfusion solutions were mixed with normal efferent blood, as the pressure increase due to the perfusion was moderate (see results)

Capillary perfusions To increase the capillary hydrostatic pressure the perfusion rate of the star vessel has to be drastically increased or else a smaller area should be used with unchanged perfusion rate The latter method was chosen. When a straight portion of a proximal tubule was found the perfusion pipette was inserted into a capillary branch supporting the tubule Special care was taken not to damage the tubule by the perfusion pipette

Interstitial perfusions The interstitium was perfused by a flat insertion of a cannula just under the capsule As soon as the tip passed the capsule a bulging of the surface indicated a successful puncture Hyperoncotic subcapsular perfusions were kept at a low hydrostatic pressure by making capsular holes on the other side of the perfused tubule This also served to direct the subcapsular fluid around the tubule In the experimental group where the interstitial hydrostatic pressure was increased, a high perfusion rate was used (500–1 000 nl min) A considerable capsular leakage from the puncture hole occurred and the capsule showed a tendency to loosen from the kidney surface whereby the perfused area increased

Measurements of hydrostatic pressure A modified servo-nulling system, in principle described by Wiederhielm *et al* (1964) was used (Instrumentation for Physiology and Medicine San Diego California) The micropuncture pipettes used for pressure recording (3–6 μm outer diameter) were filled with stained 0 5 M NaCl solution. When measurements were made in small peritubular capillaries the risk of touching the *tip to the walls was apparent. The criteria for satisfactory recording was a pulsation similar to that of recordings from large vessels.* In vitro calibrations verified the reproducibility of the system.

Fig. 1 illustrates the relative positions of the cannulae

Results

The results from all 3 groups are summarized in Table I The determined reabsorption rate (J) of fluid from a tubular segment during perfusion was compared with that obtained during normal blood flow It might be expected that the experimental maneuvering or other factors could cause an unspecific difference between control and experimental condition The succession between the two was therefore changed randomly in all but Group II In that group it was considered to be more important to check for irreversible damage of the epithelium by an immediate measurement under normal blood perfused condition

Group I Star vessel perfusion

It is noteworthy that low oncotic pressure perfusions did not have an influence on reabsorptive rate (Fig. 2) It should also be noted however that the load of protein in the capillaries was probably only slightly decreased by this procedure as indicated by a relative modest hydrostatic pressure increase in the efferent arteriolae (Table II)

The significant increase in reabsorptive rate (about 20 of control) when the perfusion

TABLE I Summary of data for all groups

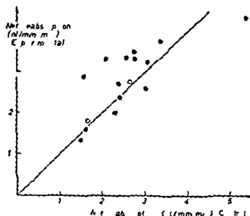
Group	Perfusate	Perfusion rate nl/min	Jv nl/mm min Control ±S D	ΔJv nl/mm min Perfusion control	Statistics	Hydrostatic pressure mm Hg
I Star vessel perfusion	a) Ultrafiltrate	125	-16±0 62	0 41±0 74	NS	Control 12 9±2 6
		250	(21)	0 27±0 78	NS	ΔP_{125} +1 7±1 3
	b) 10 g HSA	?50	2 83±1 03	0 48±0 48	S	ΔP_{250} +3 0±1 7
			(16)			(n = .0)
II Capillary perfusion	a) Ultrafiltrate	125	2 93±0 87	−1 85±1 19	S	*Control 10 9±1 9
		250	(?5)	−2 29±1 15	S	ΔP_{125} +3 0±1 8
	b) 5 g HSA	125	2 99±0 83	−1 77±1 25	S	ΔP_{2} +5 1±2 7
		250	(39)	−2 12±1 05	S	(n = 34)
	c) Ultrafiltrate Decapsulated	250	2 9 ±0 85 (?1)	−1 40±0 99	S	
III Subcapsular perfusion	a) 5 g HSA	500	2 74±1 00 (?1)	0 06±0 67	NS	
	b) 10 g HSA	500	3 33±0 69 (20)	−0 21±0 73	NS	
	c) 1 g HSA *elevated* pressure	500–*1 000*	2 80±0 70 *(27)*	−0 58±0 44 $\Delta Jv/\Delta P$ = −0 10	S	2–10 mm Hg mean pressure 6 mm Hg

Number in parentheses = number of punctured tubules S indicates that the deviations were significantly different from zero ($p < 0\ 01$) according to the Student t test Separately measured (Table II and III)

solution was 10 g / HSA (corresponding to an oncotic pressure of 60 mm Hg according to Landis and Pappenheimer 1963) (Fig 3) is in the order of what would be expected from the recently determined effective hydraulic conductivity (Schnermann Persson and Ågerup 1974) On the other hand it cannot be excluded that through some unknown mechanism the ultrafiltrate used enhanced the reabsorptive rate as indicated by the results from the colloid free perfusion solution (Group Ia) If the increase in the reabsorptive rate is com pared to the oncotic pressure difference between the HSA and ultrafiltrate solution a much less effect is derived

Group II Capillary perfusion

When the capillaries were perfused close to the tubule where the reabsorptive rate was meas ured a large decrease in reabsorption was noted (Fig 4) This effect was not influenced by the addition of HSA to the perfusate (Fig 5) or by the removal of the capsule (Fig 6) The characterizing feature of this experimental procedure is the flow dependency The hydrostatic pressure during perfusion increased 3 and 5 mm Hg from a control of 10 9 mm Hg (Table III) This increase in hydrostatic pressure is however much lower than the in crease in oncotic pressure of the perfusate when HSA was added (about 25 mm Hg Landis and Pappenheimer 1963) It thus seems as if this experimental condition induces a depres sion in the reabsorptive rate that is independent of at least the oncotic pressure During the experiments it was noted that a further increase in perfusion rate sometimes contin uously reversed the direction of the net reabsorbed fluid so that the droplet slowly increased in volume



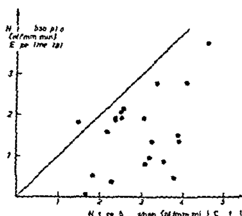
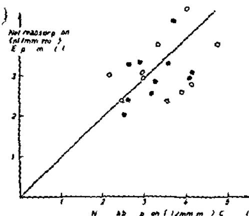


Fig 6 Reabsorptive rates measured during perfusion of the capillary with ultrafiltrate on decapsulated kidneys at 250 nl/min (Experimental) plotted against reabsorptive rates measured on the same tubular segment under normal blood perfused condition (Control) The line drawn is that of identity

Fig 7 Reabsorptive rates measured during subcapsular perfusions with 5 g HSA Under low hydrostatic pressure at a rate of 500 nl/min (Experimental) plotted against reabsorptive rates measured on the same tubular segment under normal subcapsular conditions (Control)

1 000 nl/min drastically decreases the reabsorptive rate To achieve a significant change in the blood composition a perfusion rate of 250 nl/min was chosen The oxygen content of the perfusion solution was low but mixing with fully oxygenated blood seems to have guaranteed the oxygen supply The decrease in hematocrit might also have been a source of error but a perfusion with a solution containing red cells should involve technical problems difficult to overcome



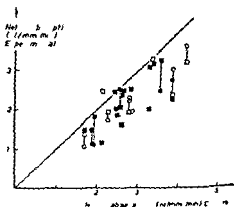


Fig 8 Reabsorptive rates measured during subcapsular perfusion with 10 g HSA under low hydrostatic pressure at a rate of 500 nl min (Experimental) plotted against reabsorptive rates measured on the same tubular segment under normal subcapsular conditions (Control) Filled symbols indicate that the experimental measurement preceded the control measurement The line drawn is that of identity

Fig 9 Reabsorptive rates measured during subcapsular perfusion with 1 g HSA under high hydrostatic pressure at rates of 500 nl min (○) and 1 000 nl min ( ) (Experimental) plotted against reabsorptive rates measured on the same tubular segment under normal subcapsular conditions (Control) Filled symbols indicate that the experimental measurement preceded the control measurement. The line is that of identity

TABLE IV A comparison between the hydrostatic pressure of the interstitium and that of the droplet (n = 13)

	Interstitial perf pressure mm Hg	Intradroplet pressure perfusion mm Hg	Intradroplet pressure control mm Hg	Intradroplet, pressure diff perf contr mm Hg
Mean ±S D	6 0±2.8	14 5±3 8	14 5±4 7	0 0±3 9

The increase in tubular fluid absorption when the peritubular capillary protein concentration was raised by means of microperfusion is in good agreement with earlier reports (Spitzer and Windhager 1970 Brenner and Troy 1971) However the failure in demonstrating the reverse effect when lowering plasma protein concentration is not readily explained and is in contrast to earlier reports The comparison is only justified on a qualitative level as these authors generally perfused at a higher rate and/or with other types of protein free solutions

When the capillaries were perfused close to the site of reabsorptive rate measurement the hydrostatic pressure in the star vessel was only slightly elevated but the capillary pressure close to the droplet was increased by as much as 5 mm Hg or 50% of the control This experimental procedure caused a drastic decrease in the reabsorptive rate (about 60 of control) This effect might have been caused by firstly the absence of colloids in the perfusion solution secondly an increase in interstitial hydrostatic pressure thirdly development of local hypoxia or cooling and fourthly microdamage due to the high flow rate (Bank Aynedjian and Wada 1972) The first possibility was tested in another set of experiments by adding 5 g % HSA to the perfusion solution The same protocol was followed and the results indicate that there was no difference in the reabsorptive rate irrespective of whether or not the perfusion solution contained colloids To test the second possibility the same experimental protocol was carried out on a kidney where the capsulae was removed This maneuver also failed in explaining the possible cause of the decrease in reabsorptive rate The last two possibilities were not tested in this study but a combination of the two seems likely On 6 occasions the reabsorptive rate was measured continuously during and after perfusion These determinations showed that the reabsorption returned to normal values immediately after perfusion was stopped This indicates that the effect was not caused by an irreversible damage to the epithelium.

As capillary perfusions are associated with not only removal of red cells but probably also other nutritive and hormonal agents necessary for normal tubular function another perfusion technique was introduced By introducing the pipette in the subcapsular space the part of the tubules under study can be superfused with different solutions under varying pressure conditions without affecting normal blood supply It is assumed that this technique makes it possible to reach the tubule with the perfusate directly on the basal side However the results when perfusing under low hydrostatic pressure with solutions containing a high protein concentration seem to indicate that this was not the case Although it has been reported that the subcapsular space is in close contact with the lymph space (Wolgast 1973) direct evidence is lacking on the subject of the connection between the subcapsular space

and the basal side of the tubule It thus seems in this study that the existence of HSA in the subcapsular space has no acute effect on reabsorptive rate On the other hand when increasing the hydrostatic pressure in the subcapsular space a significant reduction in re absorptive rate was noticed Factors that might have influenced this result in such a way as to underestimate the importance of hydrostatic pressure are at least twofold Firstly the change in transmural pressure was lower than predicted due to an increased intradroplet pressure Secondly the interstitial tissue did not transmit pressure uniformly around the tubule Against the first statement speak the measurements presented in Table IV On the other hand it cannot be excluded that other factors secondary to a pressure increase might have given cause to an overestimation of the influence of the hydrostatic pressure An example of such a factor is the possibility that the perfusion reduced the delivery of nutrient agents from the blood stream to the tubular cells However in so much as the colloid perfusions had no effect on reabsorptive rate although the perfusion rates were similar this explanation seems unlikely

It is impossible to predict the extent to which peritubular pressure changes can influence reabsorptive rate This is mainly due to the many unknown qualities of the active transport processes However as judged from the recent reports on the effective hydraulic conductivity of the proximal tubule (0 03 nl/mm min Hg) determined by changing oncotic and hydrostatic pressures in the tubular lumen (Schnermann Persson and Ågerup 1974) an estimate of the influence of passive forces on reabsorptive rate can be calculated In Group I the decrease or increase in oncotic pressure is about 20 mm Hg during the high perfusion rate The expected effect would then be about 0 03 20 = 0 6 nl mm^{-1} min^{-1} This was the case during hyperoncotic perfusions but not during hypooncotic ones indicating a possible compensatory response on reabsorptive rate from the active part when low colloid solutions were used In Group II the same line of argument yields an oncotic pressure decrease of about 25 mm Hg and a hydrostatic pressure increase of 5 mm Hg together a reduction in pressure difference of about 30 mm Hg The corresponding depression would be about 0 03 30 = 1 nl mm^{-1} min^{-1} The effect noticed was about twice that expected indicating a significant influence on the active component and/or an increase in epithelial hydraulic conductivity Finally the results obtained when perfusing the subcapsular space can only be discussed in terms of a hydrostatic pressure dependency on the reabsorptive rate as the hyperoncotic perfusions did not influence the reabsorptive rate A mean increase in hydrostatic pressure of 6 mm Hg should have resulted in a decreased reabsorption of about 0 2 nl mm^{-1} min^{-1} but the effect was three times higher On the other extreme if the reabsorption is completely dependent upon the hydrostatic pressure difference between lumen and interstitium (with a pressure difference of about 15 mm Hg under these experimental conditions) the effect would have been in the order of three times that found

The results of the present study indicate that there is no simple relation between peritubular pressure conditions and tubular reabsorption It appears as the reabsorptive rate is little sensitive for oncotic pressure changes carried out in the subcapsular space or wide interstitium Although changing the capillary physical forces leads to a prompt response on the absorptive rate this effect does not seem to be transmitted via a wide interstitium Electronmicroscopical pictures of the renal cortex show that a significant part of the basal

side of the tubule is in close contact with the capillary wall. Furthermore, the capillary area facing the tubular area contained more frequently pores with dimensions of 300–500 Å (Pedersen and Maunsbach, 1973). In essence it is possible that there exists a direct pathway for fluid to be transported from the intercellular space over the combined basement and capillary membrane into the capillary lumen. That membrane might show other characteristics with respect to hydraulic flow than does the basement membrane alone. With reservations for the limitations of the technique it seems as if some of the results favor the hypothesis of a direct transport route over the combined basement and capillary membranes. Primarily, the lack of effect when perfusing the subcapsular space with a hyperoncotic solution indicates that the major fraction of the plasma proteins is unable to attract fluid from the intercellular space. Regardless of whether this is due to a very low reflection coefficient for albumin over the basement membrane or to a restriction for the molecule in the ground substance, the function of interstitial protein is not evident. On the other hand, high concentrations of albumin in the capillary increased reabsorption significantly. Two mechanisms are possible for explaining that. There was either a direct effect over the capillary basement membrane or an indirect effect via a hydrostatic pressure decrease in the wide perfused interstitium. The latter explanation seems less probable in view of the modest influence of the reabsorptive rate observed when increasing the hydrostatic pressure in the subcapsular space. Secondly, when the capillaries were perfused under high hydrostatic pressure and flow rate, the effect of the development of microdamage should have been abolished when the capsule was removed. This indicates that microholes are open to the intercellular space and not primarily to the wide interstitium. Whether this reaction of the capillary basement membrane has any physiological meaning is impossible to say but the fast recovery (within seconds) is striking.

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Acta physiol scand 1975 93 195–201

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Observations of the Early Diuretic Response after Intravenous Administration of Bumetanide and Furosemide in Dogs

By

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Received 5 July 1974

Abstract

OLSEN U B *Observations of the early diuretic response after intravenous administration of bumetanide and furosemide in dogs* Acta physiol scand 1975 93 195–201

A transient natriuretic peak was observed in dogs the third minute after the i v administration of the highly active diuretics bumetanide and furosemide The peak is dependent on the sodium balance such that it is potentiated during positive sodium balance and is not observed in sodium depleted dogs A relationship exists between the simultaneous occurrence of the peak and abolition of the cortico-medullary electrolyte gradient.

In 1972 Østergaard *et al* from our laboratory described the pharmacological properties of a new highly potent diuretic bumetanide synthesised by Feit (1971) The activity profile of this new compound is similar to that of furosemide

In preliminary experiments the times of maximum diuretic effect with furosemide and bumetanide were compared in dogs Using 5 min collection periods an early diuretic peak was observed in some dogs with both substances before the actual diuretic maximum The purpose of this study is to attempt to clarify the nature of this initial peak

Methods

Unanesthetised female dogs were fasted for 16 h prior to the experiments with free access to water or 1 sodium chloride solution, where indicated

(1) In one series using normal dogs the substances were administered i v and the urine was collected via catheter at the following intervals 0–5 5–10 10–15 15–30 30–45 45–60 60–1 0 and 1 0–180 min

(2) To obtain urine collections of shorter interval 5 dogs were ureterostomized by suturing the site around the inlet of the urethra into the vesica to the abdominal wall in front of the floor of the pelvis Operations were performed on dogs 2 weeks prior to the first experiment The site around the orifice of the fistula was coated with lanolin ointment to avoid skin irritation

(2 A) *Dogs kept under normal conditions* After control collection of urine for 5–10 min these ureterostomized dogs which were on a standard diet with free access to water were given the drugs intravenously and the urine was subsequently collected at one minute intervals for a total of 40 min

(2 B) *Dogs in positive sodium balance* Identical experiments were performed on the same dogs which had been brought into positive sodium balance by having free access to 1% sodium chloride instead of water for 3 days prior to the experiments

(2 C) *Sodium depleted dogs* Further the same dogs were depleted of sodium by giving daily oral doses of 0 25 mg/kg bumetanide and free access to water for 3 days prior to the experiments

In each method the i v administration of the substance was made over approximately 30 s with the standard dosages of furosemide and bumetanide being 2-2 5 mg/kg and 0 25 mg/kg, respectively

Other dogs brought into the respective sodium balances were anesthetised with pentobarbital and the kidneys removed for slice analysis of the sodium and urea content per wet weight of cortex medulla and papillary regions The slices were homogenized in distilled water, centrifuged and the supernatant used for analysis

Analyses Sodium and potassium were determined by flame photometry The osmolarity of the urine was measured with an electronic semimicro osmometer (Knauer Model M) and urea was determined by the Berthelot reaction (Merckotest No 3334)

Statistical significance was calculated according to Student s t test

Results

Fig 1 shows the sodium excretion after i v administration of furosemide (2 5 mg/kg) to surgically unprepared dogs kept under normal conditions An initial peak of sodium excretion is observed within the first 5 min in 2 dogs with maximum sodium excretion lower than 0 05 meq/kg/min

The initial sodium peak was more clearly demonstrable in ureterostomized dogs with one minute urine collections Preliminary experiments with furosemide in 1 dog kept under normal conditions and in 2 dogs brought in positive sodium balance showed a distinct initial sodium peak which was more carefully investigated in dogs dosed with bumetanide

Fig. 2 shows the results of one such experiment in a dog brought in positive sodium balance The first minute after dosing with bumetanide was usually anuric Thereafter the initial sodium peak followed reaching the maximum during the third minute after drug administration after which the natriuresis was again reduced and remained lower between the 5th and 7th min, to increase again to a maximum about 20 min after dosing The initial peak constantly refers to the excretion of sodium, while the volume of urine and the excretion of potassium occasionally parallel the excretion of sodium

Table I summarizes the results obtained with bumetanide In experiments with dogs kept under normal conditions the initial peak was only observed in a few experiments Mean values demonstrate an initial rise in sodium excretion within the first 3 min followed by no change in the 3rd to 6th min before a second rise in sodium excretion

In individuals with positive sodium balance the initial peak of sodium excretion was always observed in the 3rd min after bumetanide administration and was roughly of the same magnitude as the later maximum sodium excretion The initial sodium peak was not observed in sodium depleted individuals as these dogs showed a gradual increase in sodium excretion to a diuretic maximum about 20 min after drug administration

The control sodium excretion in dogs in positive sodium balance is significantly ($p < 0.02$) higher than the sodium excretion in dogs kept under normal conditions as is the sodium excretion ($p < 0.01$) in the 3rd min after dosing with bumetanide No significant difference has been found in the later course of sodium excretion between these 2 groups

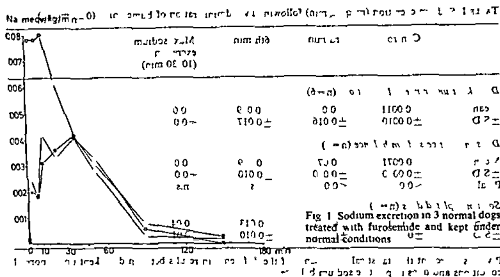


Fig 1 Sodium excretion in 3 normal dogs treated with furosemide and kept under normal conditions

The relationship between the control sodium excretion and the 3rd min sodium excretion in the different sodium balances is depicted in Fig 3 These results show a statistically significant dependence with a correlation coefficient r = 0 815 with 95% confidence limits (0 555–0 930) (Hald 1948)

Examination of the osmotic pressure of the urine (Table II) shows that the most significant changes occur within the first minutes after furosemide or bumetanide administration Here again the most important quantitative changes occur the third minute after drug administration i e the same as the initial peak of sodium excretion Also the minimum osmotic pressure of the urine and the maximum diuretic response both occur 10–30 min after drug administration It is of interest to note the wide variance in control urine osmolarity in dogs with positive sodium balance Acute saline loading normally decreases urinary osmolarity (Early and Friedler 1965)

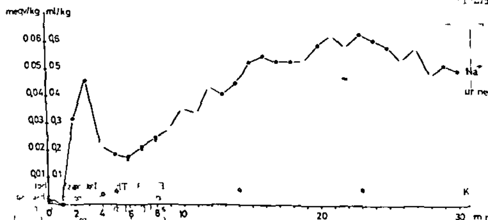


Fig 2 Excretion of sodium potassium and urine in an ureterostomized bumetanide treated (0 25 mg kg i v) dog in positive sodium balance Experiment no 15 dog weight 20 kg

TABLE I Sodium excretion (meq/kg/min) following i v administration of bumetanide (0 25 mg/kg)

	Control	3rd min	6th min	Max sodium excretion (10–30 min)
Dogs kept under normal conditions (n = 6)				
Mean	0 0011	0 037	0 039	0 07
±S D	±0 0010	±0 016	±0 017	±0 02
Dogs in positive sodium balance (n = 7)				
Mean	0 0071	0 07	0 039	0 07
±S D	±0 0040	±0 020	±0 010	±0 02
P value	<0 02	<0 01	n s	n s
Sodium depleted dogs (n = 4)				
Mean	0 000	0 004	0 013	0 045
±S D	±0	±0 007	±0 010	±0 01

P values represent the statistical significance of the difference in results between dogs kept under normal conditions and dogs in positive sodium balance

With increased diuresis the excretion of urea is known to be briefly elevated as a result of the wash-out of accumulated urea in the medulla (Ullrich and Jarausch 1956 Shimizu *et al* 1969) Here this wash-out of urea both in dogs with positive sodium balance and in sodium depleted dogs (Table III) is found to result in a peak during the first minutes more or less together with the initial sodium peak

Renal tissue slice analysis (Table IV) shows that dogs kept under normal conditions as well as dogs in positive sodium balance display similar well developed cortico medullary electrolyte gradients and demonstrate similar sodium and urea contents/g wet weight in medullary and papillary regions Sodium depleted dogs have a greatly reduced cortico-medullary electrolyte gradient Dogs in positive sodium balance demonstrate a total abolition of the cortico-medullary electrolyte gradient 15 min after the administration of bumetanide

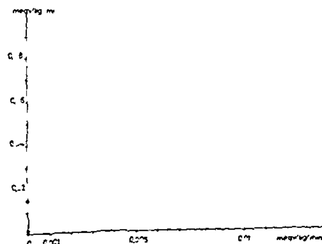


Fig. 3 The relationship between control sodium excretion (abscissa) and third minute sodium excretion (ordinate) in bumetanide treated (0.25 mg kg i v) dogs.

TABLE II Urine osmolarity (mosm/l) following i v administration of diuretics Experiments were performed with bumetanide (0 [illegible]5 mg/kg) except for experiments numbers 1 8 and 9 which were performed with furosemide (2 mg/kg)

Experiment no	Control	2nd min	3rd min	6th min	Under max sodium excretion
Dogs kept under normal conditions					
1	1 250	1 115	565	345	[illegible]5[illegible]
4	1 375	1 100	460	[illegible]80	260
5	1 400	875	455	300	[illegible]75
6	1 0[illegible]0	—	760	330	275
Dogs in positive sodium balance					
8	895	6[illegible]0	350	80	255
9	5[illegible]0	5[illegible]0	350	310	300
11	1 150	805	460	3[illegible]0	280
12	635	510	375	330	300
13	>1 600	845	565	375	3[illegible]0
14	>1 600	1 510	642	360	315
15	1 065	880	478	—	310
16	[illegible]1[illegible]4	2 040	980	—	316
Sodium depleted dogs					
17	—	—	685	310	230
18	740	—	3[illegible]5	215	180
19	810	875	450	305	2[illegible]0
[illegible]0	9[illegible]0	—	680	350	200

Discussion

The results show that an initial sodium peak under certain conditions occurs during the development of maximum diuresis after bumetanide and furosemide The occurrence of the peak depends on the sodium balance such that positive sodium balance favours its existence while it is not observed in sodium depleted individuals

The initial sodium peak takes place within the first 5 min after drug administration and is maximal within the 3rd min It is closely time related to strong changes in urine osmotic pressure and urine urea excretion

TABLE III Urea excretion (mg/kg/min) following i v administration of bumetanide (0 5 mg kg)

Experiment no	Control	2nd min	3rd min	6th min	Under max sodium excretion
Dogs in positive sodium balance					
12	0 3	0 9	0 7	0 [illegible]	0 3
13	—	3 8	3 7	1 [illegible]	1 0
14	1 3	9	3 1	0 5	1 1
Sodium depleted dogs					
17	0 1	4	2 6	[illegible] 0	1 4
18	0 3	—	1 [illegible]	0 7	0 7

TABLE I Sodium excretion (meq/kg/min) following i v administration of bumetanide (0 75 mg/kg)

	Control	3rd min	6th min	Max sodium excretion (10–30 min)
Dogs kept under normal conditions (n = 6)				
Mean	0 0011	0 037	0 039	0 07
±S D	±0 0010	±0 016	±0 017	±0 02
Dogs in positive sodium balance (n = 7)				
Mean	0 0071	0 072	0 039	0 07
±S D	±0 0040	±0 0?0	±0 010	±0 02
P value	<0 02	<0 01	n s	n.s
Sodium depleted dogs (n = 4)				
Mean	0 000	0 004	0 013	0 045
±S D	±0	±0 007	±0 010	±0 01

P values represent the statistical significance of the difference in results between dogs kept under normal conditions and dogs in positive sodium balance

With increased diuresis the excretion of urea is known to be briefly elevated as a result of the wash-out of accumulated urea in the medulla (Ullrich and Jarausch 1956 Shimizu *et al* 1969) Here this wash-out of urea both in dogs with positive sodium balance and in sodium depleted dogs (Table III) is found to result in a peak during the first minutes more or less together with the initial sodium peak

Renal tissue slice analysis (Table IV) shows that dogs kept under normal conditions as well as dogs in positive sodium balance display similar well developed cortico medullary electrolyte gradients and demonstrate similar sodium and urea contents/g wet weight in medullary and papillary regions Sodium depleted dogs have a greatly reduced cortico-medullary electrolyte gradient Dogs in positive sodium balance demonstrate a total abolition of the cortico-medullary electrolyte gradient 15 min after the administration of bumetanide

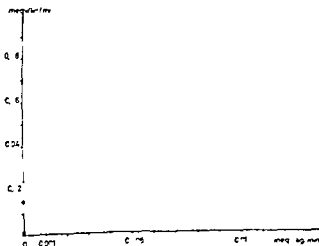


Fig. 3 The relationship between control sodium excretion (abscissa) and third minute sodium excretion (ordinate) in bumetanide treated (0 5 mg kg i v) dogs

The results partly support the concepts of Stowe and Hook (1970) and Stowe (1972) Using anesthetised hydropenic dogs they found that abolition of the cortico-medullary electrolyte gradient after furosemide to be the consequence of increased water absorption in medulla and not of the wash-out of sodium in the urine The results lend support to the existence of a sodium wash-out in the urine after bumetanide (and furosemide) although it seems to be quantitatively determined by the sodium balance

Conclusion

The early transient natriuretic peak observed the third minute after intravenous administration of bumetanide (and furosemide) is closely related to the simultaneous abolition of the cortico-medullary electrolyte gradient The peak therefore seems to be the result of a wash-out of medullary papillary sodium depots

The author is greatly indebted to Mr M P Magnussen and Mrs E. Eilertsen for having performed the analytical part of the work Further Prof E A Martelli and Dr Thomas E. Donnelly Jr are thanked for help in preparing the manuscript. Mr H Engberg Petersen is thanked for having calculated the correlation-coefficient.

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Acta physiol scand. 1975 93 202-205
From the Institute of Physiology University of Lund Sweden

Atropine Sensitivity of the Rat Urinary Bladder during Nerve Degeneration

By

MATS ELMÉR

Received 8 July 1974

Abstract

ELMER, M *Atropine sensitivity of the rat urinary bladder during nerve degeneration* Acta physiol scand 1975 *93* 202–205

The pressure response of the rat urinary bladder to electrical stimulation of the pelvic nerve at a low stimulation frequency was found to be almost completely atropine resistant. However the response to stimulation of the degenerating pelvic nerve 0–30 h after section of the nerve postganglionically using maximal stimulation frequency was totally abolished by atropine or the parasympatholytic agent Hoechst 9980 The responses were not affected by hexamethonium or dihydroergotamine but were potentiated by eserine The non-adrenergic portion of the bladder response to stimulation of the degenerating hypogastric nerves was also abolished by atrop ne or Hoechst 9980 It is concluded that the transmitter activat ing the detrusor muscle at stimulation of the pel ic nerve or the non adrenergic part of the hypogastric nerve probably is acetylcholine

Key wo ds Atropine sens tivity nerve degenerat on urinary bladder

The excitatory effect of exogenous acetylcholine on the detrusor muscle is totally abolished by atropine while the response to stimulation of the parasympathetic nerves is almost com pletely atropine resistant (Langley and Anderson 1895) It has been proposed that the motor nerves of the bladder are non-cholinergic in the dog (Henderson and Roepke 1934) in the guinea pig, cat and rabbit (Ambache and Zar 1970) and in the guinea pig and rat (Dumsday 1971 Burnstock *et al* 1972) However the vesical response to transmitter leaking from degenerating nerve endings causing degeneration contractions in the rat is completely abol ished by atropine (Elmer 1973)

In the present investigation the atropine sensitivity of the response to electrical stimula tion of degenerating motor nerves to the bladder was studied

Methods

17 male albino rats of the Wistar strain weighing about 400 g were used

In 9 rats a postganglionic parasympathetic denervation was performed unilaterally by extirpation of the left pelvic ganglion. In the male rat the pel ic plexa form distinct gangl a located on the lateral surface

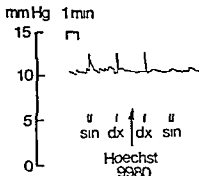


Fig. 1 Pressure responses of the rat urinary bladder to electrical stimulation of the degenerating left pelvic nerve with 15 Hz (sin) and the normal right pel ic nerve with a single shock (dx) before and after the parasympatholytic agent Hoechst 9980 0 1 mg/kg.

of the prostate gland (Langworthy 1965) Postganglionic sympathetic denervat on was ach eved in 3 rats by section of the hypogastric nerves distal to the hypogastric gangl a The operations were made asept cally in ether anaesthesia.

0–30 h after denervation the rats were anaesthetized w th chloralose (100 mg kg) gi en through a cannula in a femoral vein after induction with ether The bladder was e posed and the ureters were l gated A glass cannula was insert d nto the bladd r through an incision in the urethra The bladder was filled w th 0 .5 ml of phys olog cal sal ne and the pressure developed by the detrusor muscle was reco ded by mea s of a transducer and a polygraph

The d stal end of the earl er cut pel ic nerve on the left s de was st mulated electricall us ng a bipolar electrode In 3 of the rats the right pel ic n rve was also stimulated after section of the nerve distal to the pel ic ganglion immed tely before the experiment. In 5 rats the left pel ic nerve was stimula ed immed ately after section The earl er cut hypogastric nerves were stimulated b laterally A Grass stimulator gi ing rectangular pulses w th a duration of . ms and of supramax mal intens ty (3 10 V) was used.

D gs The substances used were atropine sulphate Hoechst 9980 (p peridino-ethyl-d phenylacetam de) hexamethon um brom de d hydroergotamine methansulphonate and eserine sulphate The drugs were i jected through the cannula n the femoral vein

Results

Electrical stimulation of the degenerating pelvic nerve 20–30 h after the postganglionic section caused contraction of the detrusor muscle Maximal pressure response was obtained with a stimulation frequency of 15 Hz for 10 s which increased the intravesical pressure 0 5-2 5 mm Hg from a resting level of about 10 mm Hg. The response to stimulation was totally abolished by 0 1–1 mg/kg of atropine The parasympatholytic agent Hoechst 9980 (Schaumann and Lindner 1951) had the same effect (Fig. 1)

After injection of 0.2 mg/kg of eserine the response was increased about 3 times The response was not affected by dihydroergotamine 2 mg/kg.

The normal pelvic nerve was stimulated with a frequency of 1 Hz for 10 s or with single shocks which increased the bladder pressure 1 5–4 mm Hg. The responses were not affected by previous injection of 0 1 mg/kg of atropine or Hoechst 9980 and only slight y reduced by 1 mg/kg in some rats The effect of Hoechst 9980 on the response to stimulation of the normal right pelvic nerve and the degenerating left pelvic nerve in the same rat is shown in Fig 1

Eserine increased the response to stimulation of the normal pelvic nerve about 3 times Dihydroergotamine 2 mg/kg, or hexamethonium, 10 mg/kg, did not affect the responses.

Stimulation of the degenerating hypogastric nerves with 15 Hz for 10 s caused a contraction of the detrusor muscle increasing the bladder pressure 0.5–2 mm Hg. The response was totally abolished by atropine or Hoechst 9980 in a dose of 0.1 mg/kg.

Discussion

When the normal pelvic nerve of the rat is stimulated using maximal frequency the bladder response is reduced by about 60% after atropine (Elmér unpublished observation). In this investigation however the normal pelvic nerve was stimulated at very low frequencies in order to get small pressure responses comparable to the responses to stimulation of the degenerating nerve. These small contractions of the detrusor muscle were almost completely atropine resistant, while the responses to stimulation of the degenerating nerves with maximal frequency were totally abolished by atropine or the specific parasympatholytic agent Hoechst 9980 (Emmelin and Stromblad 1957). This indicates that the transmitter in pelvic nerve-bladder transmission probably is acetylcholine which is further supported by the finding that the responses were potentiated by eserine.

The ganglionic blocking agent hexamethonium did not affect the responses indicating that the nerves stimulated were postganglionic. Although the rat detrusor muscle contains excitatory α adrenoceptors (Elmér 1974) the α receptor blocking agent dihydroergotamine had no effect, suggesting that the responses were not caused via an adrenergic transmission.

The non adrenergic portion of the response to hypogastric nerve stimulation found in the rat is reduced by about 60% after atropine (Elmer unpublished observation). The response to stimulation of the degenerating hypogastric nerves was totally abolished by atropine or Hoechst 9980 suggesting that the non adrenergic part of the hypogastric nerve is cholinergic.

To explain atropine resistant mechanisms Dale and Gaddum (1930) suggest that acetylcholine is liberated by the nerve endings in such an intimate relationship to the receptor that atropine cannot prevent its access. A similar theory is proposed for the urinary bladder of the dog and rabbit (Ursillo and Clark 1956) and rat (Chesher 1970).

In the present investigation the degenerating nerve fibres were stimulated at a time after denervation when the distance between nerve terminal and receptor may begin to increase and the acetylcholine content of the nerve to decrease at least according to studies in somatomotor nerves (Reger 1959, Lissák *et al.* 1939). The access of atropine to the receptor sites may thereby be facilitated during degeneration of the nerve. The atropine sensitivity of the responses to stimulation of the degenerating nerves found in this study may thus be consistent with the "proximity theory" of Dale and Gaddum.

This work was supported by grants from the Medical Faculty, University of Lund. Hoechst 9980 was kindly supplied by Svenska Hoechst AB, Stockholm.

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Acta physiol scand 1975 93 206–219
From the Department of Chemistry Karolinska Institutet Stockholm Sweden

A Compact Perfusion System for Studies on the Control of Metabolic Processes in Isolated Organs from Small Animals

By

HÅKAN ERIKSSON

Received 9 July 1974

Abstract

ERIKSSON H *A compact perfusion system for studies on the control of metabolic processes in isolated organs from small animals* Acta physiol scand 1975 93 206–219

A compact apparatus for perfusion of isolated organs from small animals is described The system is based on a module principle making it useable both in low and high pressure perfusions 40–80 ml of heparinized diluted rat blood used as liver perfusion medium is recirculated in the system which is temperature controlled by means of a water jacket A new type of capillary membrane oxygenator is used for oxygenation The system permits rapid measurements of perfusate flow vascular resistance bile production (when the isolated liver is used) urinary production (when the isolated kidney is used) gas tensions oxygen consumption pH and perfusion pressur Descriptions of the techniques used for operation and perfusion of isolated livers and kidneys from rats are given By the generally accepted criteria of viability the isolated rat liver remains almost normal for the test period of 3 h Liver functions were normal as judged by formation of glucose respiratory rate urea and bile production and gross morphology There was very little leakage of liver enzymes into the perfusate The transformation and elimination of labelled corticosterone were studied to test the steroid metabolizing activity of the isolated rat liver preparation

The understanding of metabolic processes at a molecular level in subcellular organelle preparations has reached a stage where many anabolic and catabolic pathways have been defined with respect to reaction sequences subcellular localization and control by key enzymes This has made it possible to study more complex biochemical model systems in which the interaction of such processes in intact organs may be evaluated

Among such biochemical systems the isolated perfused organ has an important position since it combines in many respects the advantages of *in vivo* and *in vitro* experiments

Technical aspects of perfusion studies have been improved and simplified during recent years so that many objections to this method of study based on the poor condition of the perfused organ have been overcome

However a close scrutiny of available methods of organ perfusions that might be used in studies of steroid metabolism revealed that further improvements in several major aspects

Abbreviations Systematic name of the steroid referred to in the text by trivial name is as follows corticosterone 11β 1-dihydroxy-4 pregnene 3 0-dione

of the technique could be achieved The perfusion system must permit rapid measurements of variables such as concentration of certain metabolites perfusate flow vascular resistance gas exchange pH of the perfusion medium, and perfusion pressure which yield information about the condition of the organ being perfused The system should also allow wide variations in perfusion conditions to make comparisons with *in vivo* experiments possible and permit perfusion of different organs without greater changes in the set up

To fulfil these requirements a new type of versatile perfusion system has been developed This system is very compact and makes it easy to control and monitor metabolic variables it can easily be changed from low pressure perfusion to high pressure perfusion it is simple to operate assemble and clean During perfusion it is possible to change the perfusion conditions *e g* perfusate flow oxygen tension perfusate pH and perfusate temperature

This paper gives a description of the system the op rative procedures used for liver and kidney perfusions and the methods by which the liver preparation may be evaluated The system has been designed especially for studies of the regulation of steroid metabolism in the liver and the kidney from rats It may also be adaptable for studies with organs from mice

Materials and Methods

R ference compounds [1,2,6 7 ^{3}H]Corticosterone (specific activity 80 000–110 000 mCi mmole) and [4-Ckort costerone (spec fic activity 56 7 mCi/mmole) were purchased from the Radiochemical Centre Amersham England) The purity of the compounds was assayed by chromatography on Sephadex LH ‿0 (Nyström and Sjovall 1968) and by radio–gas chromatography 3α 11β,21 Trihydroxy 5α pregnan 0-one was prepared by reduction of 11β,21-dihydroxy 5α pregnan 3,20-dione (purchased from Ikapharm (Ramat Gan, P O B 31 Israel) with 3α hydroxysteroid oxidoreductase as described previously (Gustafsson and Sjövall 1968 a) A mixture of the 20β and 20α epimers of 5α pregnane 3α 11β,‿0 21 tetrol was prepared by sodium borohydride reduction of 3α,11β,21 trihydroxy 5α pregnan ‿0-one 5α Pregnane 3β 11β,‿0α(and 20β) 21 tetrol was prepared by hydrogenation of corticosterone (obta ned from Dr J Babcock) followed by reduction with sodium borohydride Professor W Klyne kindly supplied 3α,11β,‿1 trihydroxy 5β pregnan 0-one from the Medical Research Council Ste oid Reference collection

Analyt cal m thods M crochem cal reactions gas–liquid chromatography radio–gas chromatography and gas chromatography–mass spectrometry were carried out as described previously (Gustafsson and Sjovall 1968 b Eriksson and Gustafsson 1970 b)

Glucose was assayed by a glucose he okinase method (Boehringer Kit No 15994) Glutamate–oxaloacetate (GOT) and glutamate pyru ate (GPT) transaminases were determined by standard methods (Boehringer Kits No 15791 TGAE and 15978 respect vely) Urea and bilirubin were assayed by colori metric methods (Boehringer Kits No 15954 and 15944 respectively) Hemoglobin concentration was measured spectrophotometrically as oxyhemoglobin at 540 nm

Sodium and potassium concentrations were determined by flame photometry

Radioacti ity was measured in a Packard Liquid Scintillation Counter (Model 43 2) using Instagel® (Packard Co) as scintillator

All chemicals and solvents were of analytical grade.

A imal Livers and kidneys were taken from female and male Sprague Dawley rats weighing between 230–‿70 g. Prior to operation the animals had free access to food and water

Surgical technique and anatom al considerat ons

Li er perf ion. The liver is supplied *via* the portal ve n (80%) and the hepat c artery (0) but Powis (1970) us ng both routes for perfusion could not find any ad antage with th s set-up compared to portal ve n perfus on alone For this reason the present system was des gned for portal in perfusion alone

By a m dline inc s on the li er is exposed and the gastroduodenal and gastrohepatic ligaments are d ided

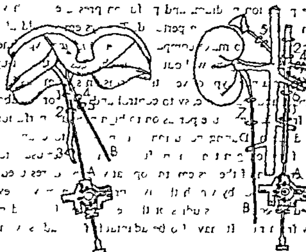


Fig. 1 Operative procedures for liver and kidney perfusions. The ligatures are placed around the following structures A. Portal vein (1 2, 3) common bile duct (4 5) B Aorta (1 2) left renal artery (3) mesenteric artery (4) adrenal branch of the right renal artery (5) left renal vein (6) right ureter (7)

after ligation of the pancreaticoduodenal vessels, with division also of the fine ligament between the liver and the vena cava The common bile duct is dissected free and a 15 cm polyethylene cannula (PE 10 or PE 50) is inserted with the end placed 3 mm from the portal hilus and secured with two ligatures (Fig. 1 A) Bile should begin to flow immediately

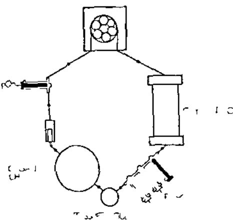
For portal cannulation two lateral incisions in the abdominal wall are made and the portal vein is exposed After distal ligation the vein is cannulated with a soft polyethylene cannula (70 1 15 mm) containing a steel mandrin and a three way stopcock. After cannulation the mandrin is removed leaving the soft cannula in place with its end about 3 mm from the point of portal vein division without damage to the vessel. When the portal cannula is filled with blood regurgitating from the liver the stopcock is turned and 1 0 ml of the perfusion medium containing 400 I U of heparin is slowly injected

The liver is then rapidly dissected out and transferred to the organ chamber The portal cannula is connected to the supply of oxygenated perfusate the stopcock is reset and perfusion is started The perfusate leaves the liver through the cut ends of the vena cava.

The whole operation procedure takes 8 10 min and the liver is deprived of oxygenated blood only for about 60-90 s. The perfusion pressure is kept at 20-25 cm H_2O to give a flow rate of 2 5-4 0 ml min/g liver

Bile samples are collected in weighed tubes every 10 min.

Kidney perfusion A vertical incision is made from the xiphoid process to the symphysis pubica followed by right and left transverse subcostal incisions from the midline into the flanks. The abdominal wall is folded back The intestines and the stomach are displaced. The peritoneum overlying the aorta and the ureters is stripped away to expose the coeliac, the superior mesenteric and the renal arteries, the renal vein and the ureters. Two ligatures are placed around the right ureter (Fig. 1 B) and it is cannulated with a 15 cm long polyethylene cannula PE 10 The urine flow starts immediately Loose ligatures are placed around the vessels shown in Fig. 1 B The right kidney is mobilized as far as possible leaving only its vascular and ureteric attachments. The arterial cannula is inserted about 5 cm distal to the right renal artery the tip of the cannula is advanced to a level 3 mm distal to the opening of the right renal artery are tied in place The proximal aortic ligatures (2) are tied. Two ml of heparinized perfusion medium is slowly infused through the arterial cannula. The kidney is transferred from the animal to the organ chamber The whole operation takes about 20 min and the kidney is deprived of oxygenated blood for about 90-120 s. The perfusion pressure is raised to 100-120 mm Hg within 60 s, giving a flow rate of 2-3 ml min/g kidney



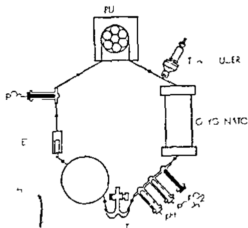


Fig 2 Circuit diagram of the perfusion system

Perf sion m dia

Lic r pe f on The perfusate consists of 20 % whole blood d luted with freshly prepared heparinized Krebs Henseleit bicarbonate buffer (Krebs and Henseleit 1932) Perfusate volumes between 40–80 ml were used Prior to perfusion 5 000 I U of heparin are added to the medium The perfusate is circulated through the apparatus for about 15–30 min to allow the system to become equ librated prior to insertion of the liver into the perfusion apparatus

K dney perf sion The perfusion medium used is a semi-synthetic one essentially the same as described by Nishiitutsuji Uwo *et al* (1967) This consists of Krebs Henseleit bicarbonate buffer made 4 5 (w/v) with respect to bovine serum albumin Before use the perfusate is filtered through a 0 22 μ m Millipore filter

Perfusion apparatus

General outline The perfusion circuit (Fig 2 and 3) is a module system consisting of twelve different parts A capillary membrane oxygenator on a rocker (A) a bubble trap (B) an organ chamber (C) a system for continuous measurement of blood gases and pH on the inlet side mounted on a pressure regulator (D) a needle valve and a flow meter to control gas flow through the oxygenator (F and E) a flow cuvette with an oxygen electrode on the outlet side of the organ (G) a transducer for measurement of perfusion pressure (H) a filter holder with a 3 way stopcock (I) a motordriven rocker for the oxygenator (J) a pump and a heat exchanger system

The apparatus is made of glass plexiglass polyethylene vinyl and silastic tubings These materials have been chosen for low toxicity ease of cleaning (or disposability) and the property of being transparent The apparatus can be assembled in less than 30 min

Oxygenator Many types of devices have been constructed to accomplish oxygenation of perfusion media In small organ perfusion systems the most frequently used oxygenator is the multibulb glass oxygenator primarily introduced by Miller *et al* (1951) and later improved by Hems *et al* (1966) Other types of oxygenating systems are represented by bubble oxygenator (Morgan *et al* 1961) the rotating chamber oxygenator (M

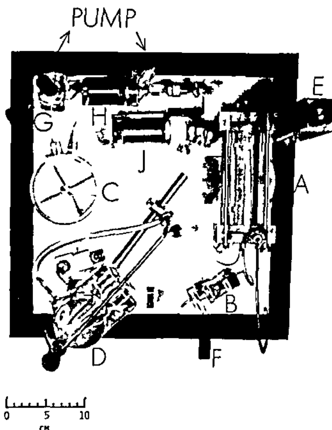


Fig 3 Photograph of the perfusion system taken from above A = oxygenator B = bubble trap C = organ chamber D = pressure regulator and unit for measurement of O_2 CO and pH E = needle valve F = gas flow meter G = flow cuvette with an oxygen electrode H = transducer I = filter holder with a 3 way stopcock J = motordriven rocker

1961) the rotating disc oxygenator (Gerber 1965) and the rotating cylinder oxygenator (Scholtz 1968) Oxygenators of this open type based on a principle of direct contact be tween the perfusate and oxygen encourages microscopic air embolism formation and de *fibrination of the blood These systems are also prone to cause denaturation of blood* proteins and damage to blood cells

To eliminate or minimize these and other adverse effects a simple oxygenator of the closed (membrane) type was developed

The construction of the oxygenator is shown in Fig 4 A central gas exchange unit is surrounded by 2 plexiglass cylinders to form a water jacket The gas exchange unit and the 2 cylinders fit into 3 grooves in 2 square plexiglass plates joined together by 4 stainless steel rods 3 silicone rubber gaskets in each end plate provide a leak free seal of the oxygena tion compartment and the water jacket

Perfusate enters the gas-exchange unit through an inflow cannula in the end plate The gas-exchange unit consists of a central plexiglass cylinder 1 0 cm in diameter mounted

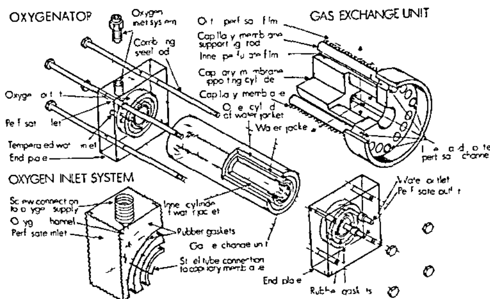


Fig 4 Schematic drawing of the oxygenator

on this are 12 thin plexiglass rods 0 5 mm from the cylinder Around these rods is wound 6 0 m of silastic tubing (i d 0 012 inches o d 0 025 inches) yielding 2 membrane surfaces one between the central cylinder and the tubing, and the other between the inner wall of the water jacket and the tubing The total membrane area formed in this way is about 120 cm The perfusate is pumped through 5 inflow channels in the central cylinder and is distributed in 2 thin layers on the inside and outside of the capillary membrane The oxygenated perfusate leaves the unit through corresponding outflow channels

One end of the capillary tubing is connected to the gas supply (O_2 CO 95 5) through an inlet system The gas flow from the main supply is controlled by a needle valve and is measured with a flow meter (Fig 3 F and E) before entering the oxygenator

The gas leaves the system through a steel tube in the endplate

The oxygenator is mounted on a small motor driven rocker the speed of which can be varied A rate of 20 cycles per minute provides gentle mixing and complete oxygenation of the perfusate

Unit for measurement of O_2 CO and pH Modified equipment provided by Radiometer (Copenhagen) was used a measuring chamber on the inlet side (DS 660 14) containing 4 ground channels for insertion of standard electrodes an oxygen electrode (E 5046) a carbon dioxide electrode (E 5036) a glass electrode (G 265C) and a salt bridge in connection with a calomel electrode (KS 67053) for measuring pH The flow cuvette for a single oxygen electrode on the outlet side (Fig 3 G) was manufactured in this laboratory The temperature is controlled by water circulating through a water jacket

The electrodes are connected to a measuring instrument (Radiometer type PHM 71)

Calibration is performed using nitrogen and atmospheric air for the oxygen electrode 2 mixtures containing 4 % and 16 % CO_2 in air for the carbon dioxide electrode and 2 buffers with pH 6.841 and 7.383 for the pH electrodes.

Pressure regulator for low pressure perfusion. The pressure regulator (Fig. 3 D) consists of a jacketed glass tube with connections to the oxygenator, the organ chamber and the heat exchanger. The level of the perfusate above the organ can be varied to obtain the desired perfusion pressure.

A small reservoir at the top serves as inlet for perfusate and outlet for overflow.

High pressure perfusion. When used for high pressure perfusion the system is modified in the following manner. The pressure regulator is excluded, and a bubbletrap and a transducer is introduced to the system. The transducer (Statham Model UC 3, Statham Inst. Inc., California 93030) (Fig. 3 H) is equipped with a blood pressure accessory (Model UBP5) which makes a continuous reading of the perfusion pressure possible. The flow cuvette with the electrodes for measurements of gas tensions in the inlet perfusate is placed between the bubbletrap and the oxygenator.

Organ chamber. The organ chamber described here (Fig. 5) was primarily designed for liver perfusions but can be used for kidney perfusions with small modifications. The organ net made of polyethylene is slightly coned to give the liver a position corresponding to that *in vivo* and rests above the funnel shaped perfusate reservoir, which is surrounded by a water jacket.

A side arm, containing the inlet for perfusion medium from the pressure regulator, a by pass connection and a Luer fitting for the portal cannula, extends from the cylindrical part of the organ chamber. This side arm also holds a stopcock through which part of the perfusion medium may be directed to a by pass circulation tunnel.

The organ chamber can be closed with a cover (see Fig. 3) which also holds a tube through which the overflow perfusate from the pressure regulator can be directed.

Filter. The perfusate outlet from the transducer is connected to a small filter holder containing a stainless steel net (150 mesh/inch) (Fig. 3 I). The filter holder is attached to a 3 way stopcock, used for continuous infusion of substances or for withdrawal of perfusate samples.

Pump. The present apparatus includes a peristaltic roller pump (Model LKB 2115, LKB Sweden) equipped with Tygon tubing. The pump is controlled by a calibrated helipot and flow is linear with increasing speed up to 70 ml/min. A pump giving a pulsatile flow (Harvard Apparatus Model 1405) may also be used.

Temperature Control System. All parts of the perfusion system are surrounded by water jackets which are connected in series. Water at 38 C is circulated through the system. The temperature of the perfusate is monitored in the pressure regulator and is kept at 37 C. An initial period of about 10 min is needed for the perfusion medium to reach 37 C.

Test of Liver Function. Following transfer of the liver to the apparatus, pH, P_{O_2} and P_{CO_2} of the inflow perfusate are measured every 10th min. The P_{O_2} of the perfusate leaving the oxygenator is maintained at 250–300 mm Hg, P_{CO_2} at 20–25 mm Hg and pH at 7.35–7.45 by adjustments of the gas flow through the oxygenator and by addition of small amounts of 0.6 N sodium bicarbonate.

ORGAN-CHAMBER

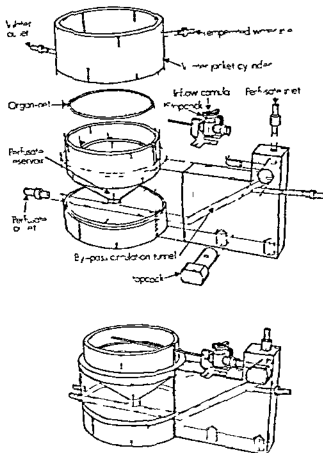


Fig. 5 The organ chamber used for isolated liver perfusion.

Assessment of liver function was based on

1 Bile production mg bile/g liver
2 Oxygen consumption, ml/min/100 g liver
3 Bilirubin concentration in the outflow perfusate mg/100 ml
4 GOT and GPT in the outflow perfusate mU/ml
5 Glucose liberation into perfusate μmole/g liver
6 Urea concentration in outflow perfusate μmole/g liver
7 Outflow and inflow potassium concentrations mM

To investigate the reproducibility with respect to these variables 5 livers from female and 5 from male rats were perfused under standardized conditions

Metabolism of corticosterone In order to investigate the steroid metabolizing activity of the rat liver in the perfusion unit two experiments were carried out.

In the first experiment the uptake and elimination of labelled corticosterone from the perfusate was studied The labelled isotope 0 5 μC of [1 2 6 7 ^{3}H]corticosterone was added to the perfusion medium in 0 5 ml of a 1% (w/v) serum albumin solution and was pre-circulated to equilibrate within the apparatus and the blood cells before the liver was inserted 500 μl of the perfusion medium were removed at intervals of 5 minutes for the first 30 min and then every 10th minute for another 30 min period The samples were centrifuged and the radioactivity was measured in the supernatant Bile was collected over six 30 min periods for a total of 180 min and 1/100 of each fraction was analyzed for radioactivity Results based on 4 such perfusions are reported here

The second experiment was performed to study the metabolic transformations of corticosterone in the female rat liver In each of 2 perfusions 5 mg of [4-^{14}C]corticosterone (0 5 × 10^6 counts/min) was used as substrate The steroid was dissolved in 50 μl of ethanol and was added to 3 0 ml of 1% (w/v) serum albumin solution The solution was given by constant infusion during 120 min at a rate of approximately 25 μl/min

Extraction and purification of biliary corticosterone metabolites Each perfusion lasted for 180 min By that time more than 95 of the administered radioactivity had been excreted into the bile The bile samples from the 2 perfusions (1 5–1 9 ml) were analyzed essentially as described previously (Cronholm *et al* 1971)

Results

The respiratory characteristics of the livers were the following at a pH of the perfusion medium of 7 35–7 45 regulated by changes of the gas flow through the oxygenator and by addition of small amounts of sodium bicarbonate the mean oxygen consumption was between 4 80 and 5 10 ml/min/100 g liver

The macroscopic changes of the liver were very small The mean output of bile was 85–90 mg bile/g liver/h during the first hour and fell to 50–60 mg bile/g liver/h at the end of the third hour (Fig. 6)

The bilirubin concentration in the perfusate rose to about 1 4 mg/100 ml during 3 h The GOT and GPT activities in the perfusate at zero time were about 15 and 2 mU/ml respectively (Fig. 6) The values increased slowly to 105 and 50 mU/ml respectively during the 3 h perfusion No changes in the potassium concentrations of the in and outflow perfusate could be detected

The net production of glucose was about 80 μmol/g liver/h during the first hour of the perfusion and fell to about 20 μmol/g liver/h during the third hour

After an initial rapid rise the urea concentration increased more slowly to about 35 μmol/g liver at the end of the perfusion

Metabolism of corticosterone The rate of disappearance from the perfusion medium of a tracer dose of labelled corticosterone was very rapid during the first 3–5 min ($t_{1/2}$ ~1 min) The elimination rate then decreased giving a half life time of about 22 min for the remaining period of perfusion (Fig. 7) Metabolites of corticosterone appeared in the bile with this latter rate

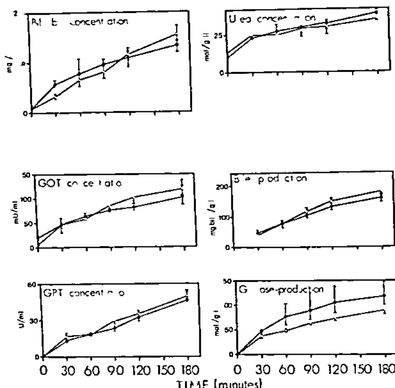


Fig. 6 Tests of liver function during perfusion Open circles represent female rat livers and closed circles male rat livers Glucose production is presented as the cumulative raise during 180 minutes Bile product on is presented as the amount (mg g liver) produced cumulatively during 180 min.

In the second experiment in which corticosterone was infused into the perfusion medium at a constant rate 92 and 96% of the infused radioactivity was recovered in the bile in the 2 perfusions 56 per cent of the biliary radioactivity consisted of monosulphurylated compounds The glucuronide and disulphate fractions contained 16 % and 26% respectively Only 2 % of the administered radioactivity was found in the unconjugated steroid fraction

Identification of metabolites of corticosterone Three main groups of corticosterone metabolites were found a) Ring A reduced metabolites with a 20-keto group b) Ring A reduced metabolites with a 20-hydroxy group c) Ring A reduced metabolites with a 15-hydroxy group

The metabolites identified by gas-chromatography and gas chromatography–mass spectrometry are listed with their relative retention times in Table I

Kidney perfusion All results concerning function and steroid metabolizing activities of the isolated perfused rat kidney will be described in detail in a separate publication

Discussion

The perfusion system described in this paper has been developed to overcome certain limitations of previously reported methods in studies on steroid metabolism. Efforts have

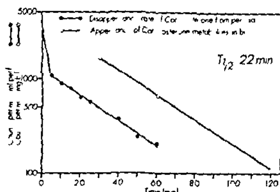


Fig 7 Disappearance rate of corticosterone from perfusate and appearance of corticosterone metabolites in bile

been made to make an apparatus which is reliable non destructive to the perfused organ and the perfusion medium simple to assemble disassemble and clean simple to hand e and modify and inexpensive to use

The most important part in an effective perfusion system is the oxygenator

4 factors of major importance for the efficiency of the oxygenator are the gas pressures the membrane material the perfusate film thickness and the flow characteristics of the perfusate film In addition low humidity and temperature of the oxygen increases oxygena tion

The oxygenator in the present system employs Silastic capillary tubing for gas transport o the perfusate pool This membrane material has previously been used in oxygenators lesigned for extracorporeal circulation in humans (Bodell *et al* 1963) The use of a capillary ubing permits a compact design and allows oxygen to be introduced into the membrane pace under high pressure with only minor effects on the perfusate flow Furthermore a arger membrane surface area per unit volume is presented to the perfusate for gas ex hange than is the case in a sheet membrane oxygenator The 2 thin perfusate films formed in each side of the coiled tubing are kept in turbulence by a slow rocking movement of the init All these factors cooperate to improve the gas exchange efficiency of the oxygenator The capacity of the oxygenator can be varied over a certain range by changing the inlet ressure and the cycle speed of the rocker With this construction it is possible to vary th ixygen tension of the perfusate leaving the oxygenator between 70 and 700 mm Hg a he same time as P_{CO} is kept at 20–30 mm Hg and the pH at 7 40

Most perfusion systems previously described require relatively large volumes of pe fusion medium This may result in a sluggish control by the liver of metabolite levels i the perfusate In the present construction the amount of perfusion medium can be varie between 25 and 100 ml This means that volumes close to the volume of the extracellula compartment of the intact rat can be used To study the viability of the organ in the presen perfusion system certain variables were monitored during the perfusion period

Minute changes in perfusate potassium and hemoglobin concentrations and in hematocri indicate a very low degree of hemolysis as a result of mechanical action of the roller pump There was very little change in O uptake throughout a 3 hour perfusion The average rate

TABLE I Relative retention times (5α-cholestane = 1 00) of silyl ethers of steroids recovered after perfusion of isolated female rat livers with corticosterone and of silyl ethers of corresponding reference compounds

Compound	SE 30	QF 1
3α 11β,21 Trihydroxy 5α pregnan 20-one	1 90	2 48
Compound A	1 92	2.48
5α Pregnane 3α,11β 20β,21 tetrol	2.28	1 68
Compound B	2.28	1 66
(3α,11β 15α,21 Tetrahydroxy 5α,14ξ pregnan 20-one)	—	—
Compound C	2.3[illegible]	2 30
11β 21 Dihydroxy 5α pregnane 3,20-dione	2 36	3 66
Compound D	2.38	3 68
3β 11β,21 Trihydroxy 5α pregnan 20-one	2.57	3 4[illegible]
Compound E	2.58	3 40
5α Pregnane 3β 11β,20β,21 tetrol	3 10	2.[illegible]4
Compound F	3 1[illegible]	2 2[illegible]
(3β 11β 15α,21 Tetrahydroxy 5α,14ξ pregnan 20-one)	—	—
Compound G	3.33	3 18

observed was 5 0 ml/min/100 g liver The values for oxygen consumption are in good agreement with those maximal values reported by Seglen (1972)

Bile production by the isolated liver is affected by a variety of factors such as perfusion pressure oxygen tension and pH of the perfusate (Brauer *et al* 1953) In the present study bile output showed an initial increase during the first 30 min followed by a slight decrease during the remaining perfusion period Simultaneously there was a slow increase in the concentrations of bilirubin and transaminases in the perfusate These changes indicate that minor cell damage occurs during the perfusion period The fall in bile output might also be explained by the elimination of bile acids initially present in the liver into bile and not replaced by enterohepatic circulation or by inadequate bile acid synthesis during perfusion

The increase in urea concentration of the perfusion medium is indicative of active amino acid metabolism by the liver This subject has been discussed in detail by Miller *et al* (1956) The net production of glucose during the whole perfusion period is probably the combined result of glycogen breakdown and gluconeogenesis

The transformation and elimination of labelled corticosterone was studied to test the steroid metabolizing activity of the liver preparation An initial rapid uptake of the steroid by the liver was followed by a slower excretion in bile Further studies are required to explain the initial uptake Possibly metabolism and excretion of endogenous steroids during the operation and transfer of the liver to the perfusion apparatus results in liberation of steroid binding sites These sites may then become rapidly occupied by part of the tracer dose of steroid added to the perfusion medium.

Previous *in vivo* studies in rats have shown a plasma half life for corticosterone averaging 30 min (Berliner and Dougherty 1960) This is comparable to the half life of 22 min found in the present study If biliary excretion is the major pathway for elimination of cortico-

sterone in the rat the half life time might be expected to be shorter in the perfused liver than in the intact rat where several organs retain the steroid and where an enterohepatic circulation may take place (Eriksson and Gustafsson 1970 a Eriksson 1971 b)

About 82% of the biliary corticosterone metabolites were mono or disulphurylated 16% were found in the glucuronide fraction and only 2% were unconjugated These findings are in good agreement with those on biliary metabolites of corticosterone in bile fistula rats (Cronholm et al 1971)

Different types of corticosterone metabolites showed a different pattern of conjugation Thus 3α(3β) 11β 15α 21 tetrahydroxy 5α 14ξ pregnan 20-one and 11β 21 dihydroxy 5α pregnane 3 20-dione constituted the major monosulphates whereas 3α(3β) 11β 21 tri hydroxy 5α pregnan 20-one and 5α pregnane 3α 11β 20β 21 tetrol were major disulphates All metabolites isolated had a 3α 5α or 3β 5α configuration a finding which is in good agreement with previous results *in vivo* (Gustafsson 1968) Thus the pattern of metabolites formed after perfusion with corticosterone agreed very well with the biliary pattern of corticosterone metabolites in bile fistula rats (Eriksson 1971 a)

The findings indicate that conditions resembling those existing *in vivo* may be achieved in the present perfusion system The system has also been shown in other experiments to be useful in studies of control mechanism of steroid metabolizing enzymes (Eriksson 1974)

I am greatly indebted to Mrs Ulla Thalin and Mr Sven Hvarvenius for excellent technical assistance

This work was supported by grants from the Swedish Medical Research Council (project No 03X 3972) the Karolinska Institutets Fonder Dr T A A Amundsons Fond and from Harald Jeanssons Stiftelse and Harald and Greta Jeanssons Stiftelse

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Acta physiol scand 1975 93 220-227
From the Department of Physiology Karolinska Institutet Stockholm Sweden

Rate Limiting Factors in Sympathetic Neurotransmitter Secretion

By

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Received 30 July 1974

Abstract

STJÄRNE L *Rate limiting factors in sympathetic neurotransmitter secretion* Acta physiol scand 1975 93 220–227

Isolated superfused field stimulated preparations of guinea pig vas deferens in which the neural stores of noradrenaline (NA) had been labelled by preincubation with tritium marked ( ) NA were used to study the factors regulating the amount of NA secreted from the nerves per applied shock The results indicate that stimulation secretion coupling in this tissue is subject to 2 different kinds of facilitation one independent and one dependent on nerve stimulation frequency Kinetic analysis of the calcium dependence of transmitter secretion after removal of α adrenoceptor mediated negative feedback control suggests that there is a definite upper limit to the amount of transmitter which can be secreted per pulse from each secretory area of the nerves The low Q_{10} value of this apparent V_{max} suggests that the rate limiting factor is non-enzymatic in nature It is proposed that the ultimately rate limiting factor in NA secretion may rather be the number of vesicles in each potential secretory area which are in a suitable position within the nerve for active participation in the secretion of transmitter

On electrical stimulation of the sympathetic nerves of isolated tissues it appears that complex factors modify the amount of transmitter secreted per applied shock (Δt) In the present paper which is based on work with isolated guinea pig vas deferens evidence is presented that Δt may vary with 2 different factors Firstly with the number of active secretory areas (varicosities?) in the nerve terminals and secondly with the amount of transmitter secreted from each individual secretory area Tentative kinetic analysis suggests that there is a definite V_m to the secretory mechanism its low Q_{10} indicates that the ultimately rate limiting factors are non-enzymatic in nature

Material and Methods

The experiments were carried out in isolated superfused field stimulated preparations of vas d from guinea pigs weighing about 300 g the methods have been described in detail elsewhere (in press a) The noradrenaline (NA) stores of the nerves were labelled by preincubation with 10 μC of ^{3}H-(−)-NA (New England Nuclear Corp) per ml Tyrode solution The r [illegible] of ^{3}H [illegible] supramaximal field stimulation (the preparation was mounted between paral[illegible] m wire (mm apart) with biphasic pulses 1 5 ms in duration was completely blocked [illegible]

was thus clearly neural in origin. Since practically all of the ^3H remaining in the tissue after extensive washing was intact ^3H NA and since 0 6 μM desipramine and 10 μM normetanephrine were added to prevent rebinding of NA (Iversen 1971) the evoked fractional rise in efflux of total ^3H

$$\Delta t = \frac{\text{Evoked rise in efflux of } ^3H}{\text{Total H in tissue at the time of stimulation}}$$

was used to measure the secretion of ^3H NA from the nerves.

The perfusion medium was Tyrode solution, in which the calcium concentration was normally 1 8 mM in some experiments it was varied stepwise to 8 4 2 and 1 mM Temperature was kept at 31 C, except in some experiments where it was lowered to 25 C or raised to 37 C

The tension of the preparation was measured by a Grass force displacement transducer and recorded on a Grass Polygraph the resting tension was adjusted to 1 g.

Results

a *Two types of facilitation of the contractile response to nerve stimulation* The preparation did not contract in response to single shocks nor to trains of supramaximal stimuli at 1 25 Hz or less the threshold was usually reached at 2.5 Hz, and always at 5 Hz. However at all frequencies a certain minimum number of pulses had to be applied before the preparation started to contract This latency corresponded to an average of 11 2±0 5 (n = 31) shocks independently of frequency from the threshold at 2 5 or 5 Hz, to 20 Hz (Fig. 1) The minimum number of shocks required to elicit contraction was often initially lower about 5 but increased with the duration of the experiment Similarly considerable hysteresis with respect to latency was observed, on stimulation with successive trains of 30–60 shocks at 2.5 5 10 20 20 10 5 and 2.5 Hz (Fig 1)

While it otherwise appeared that the same number of stimuli was required to make the preparation contract at all at frequencies ranging from 2.5 to 20 Hz, both the rate of rise and the maximum height of the contraction after this initial latency increased with stimulation frequency (Fig. 1)

b *Temperature dependence of the mechanisms for NA secretion* The curve describing the calcium dependence of the secretory mechanism in the absence of alpha adrenoceptor blocking drugs was slightly sigmoid in shape Addition of 3 6 μM phentolamine shifted the curve to the left and changed it into a perfect hyperbola, which yielded a straight line in the double reciprocal Lineweaver–Burk plot of Δt against calcium in the medium (Fig. 2) The values for Δt at 31 and 37 C were almost identical at 25 C Δt was slightly but significantly ($p < 0\ 001$) depressed altering the slope but not the intercept with the abscissa, of the straight line obtained in the Lineweaver–Burk plot (Fig 2)

c *Threshold concentration of exogenous NA required for contraction* Under the experimental conditions the threshold concentration of exogenous (−)-NA required to elicit contraction was 10–25 μM the height of contraction increased with the NA concentration up to 50 μM (Fig. 3) Beyond this concentration contractions became irregular and rhythmically oscillating.

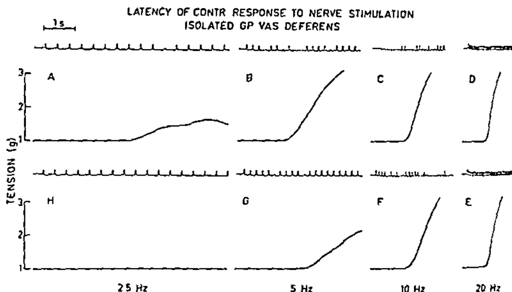


Fig. 1 Typical experiment showing latency of contraction of isolated guinea pig vas deferens in response to nerve stimulation with successive trains of 30–60 shocks delivered at 2 5 5 10 0 0 10 5 and 5 Hz (A–H) with one min intervals Ordinate Tension of preparation Sensitivity adjusted to emphasize response at low frequency peak of contraction at higher frequencies is not shown For details see Text

Discussion

a *Two types of facilitation of sympathetic neurotransmission One independent and one dependent on stimulation frequency* Independently of nerve stimulation frequency and therefore independently of the duration of stimulation a certain number of supramaximal shocks had to be applied before the preparation started to contract at all But once it started to contract both the rate of rise and the maximum height of contraction increased with nerve stimulation frequency

There is a striking parallellism between these results and those obtained in electrophysiological studies of the same tissue (Burnstock and Holman 1961 Burnstock Holman and Kuryiama 1964) In these repetitive nerve stimulation at frequencies between 2 and 15 Hz was found to cause a progressive increase in the amplitude of the initial 6 to 10 excitatory junction potentials (EJP s) While the number of shocks required to reach the fully facilitated EJP amplitude was independent of stimulation frequency the rate of rise of the amplitude of EJP s and the height of the final peak EJP level were proportional to nerve stimulation frequency The results were interpreted as evidence for facilitation of transmitter secretion *i e* that each of the initial successive stimuli caused secretion of more transmitter than the preceding one (Burnstock *et al* 1964) Two possible explanations were considered That increased secretion of transmitter was due to increase in the number of functional nerve endings or alternatively that it was due to increase in the amount of transmitter secreted from the same number of endings The first alternative which might imply that repetitive stimulation causes an increase in the number of axons invaded by action potentials

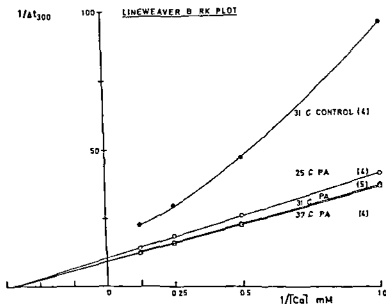


Fig. 2 Lineweaver Burk plot showing calcium dependence of Δt in control at 31 C and after disinhibition with phentolamine 3 6 μM at 25 C 31 and 37 C. Ordinate Reciprocal of fractional secretion of H NA per train of 300 shocks Abscissa Reciprocal of calcium in medium Within brackets Number of observations on each point For details see Text

(Krnjević and Miledi 1959) was considered possible (Burnstock *et al* 1964) However the second alternative increased secretion of transmitter from each of the invaded terminals was regarded as sufficient to explain the results (Burnstock *et al* 1964)

Judging from the present results both alternatives may apply since they suggest that there are 2 different kinds of facilitation of sympathetic neurotransmitter secretion in guinea pig vas deferens one independent and one dependent on nerve stimulation frequency The electrophysiological observation that 6–10 shocks independently of frequency were required to reach the fully facilitated EJP level (Burnstock and Holman 1961 Burnstock *et al* 1964) plus the present observation that a similar number of shocks independently of frequency was required to elicit contraction may imply that initially only a fraction of the potential secretory areas of the nerve terminals were activated by each nerve shock possibly due to failure of the action potentials to invade the most peripheral parts of each individual axon (Krnjević and Miledi 1959 Burnstock *et al* 1964) It is conceivable that *e g* the NA secreted from the active parts of the axon may have facilitated conduction along its more terminal parts (Burnstock *et al* 1964) gradually opening up more and more of the finest arborizations of the axon Thus the fully facilitated EJP amplitude level may possibly be reached when essentially all potential secretory areas of the nerve terminals are activated by each applied shock This seems likely in view of the fact that about the same number of shocks was required to cause contraction activation of all secretory areas by each shock may be necessary to simultaneously depolarize all muscle fibers throughout the

secretory area (varicosity?) While it is widely assumed that NA is secreted immediately from the vesicles (Smith and Winkler 1972) the size of the NA quantum is not known However there seems to be good reason to doubt that it could correspond to the entire NA content of the average nerve vesicle about 15 000 molecules (Dahlstrom *et al* 1966) In the close junctions of *e g* vas deferens with a synaptic gap of 20 nm a NA quantum of 15 000 molecules would create absurdly high local NA concentrations about 1 M (Furness 1974 Stjarne in press b) It may be significant that a NA quantum corresponding to only a few percent of the NA content of single vesicles about 400 molecules (Folkow *et al* 1967) would create a local NA concentration in these narrow synaptic gaps of about 10 μM because this happens to be close to the observed threshold of guinea pig vas deferens for exogenous NA (Fig 3) Assuming that each propagated nerve impulse after the initial latency in the normal depressed state of electro-secretory coupling in this tissue causes secretion of one such small quantum of NA from each potential secretory area (varicosity?) corresponding to an average of about 2 × 10 ⁸ of the total tissue store of NA (Folkow *et al* 1967) the apparent $V_{m\ x}$ value found in the present study suggests that the upper limit to the secretory capacity may be reached when about 20 NA quanta are simultaneously secreted per pulse from each potential secretory area

The individual varicosity (secretory area"?) of the sympathetic nerves of guinea pig vas deferens contains an average of about 1 000 NA vesicles (Dahlstrom *et al* 1966) Assuming that the propagated nerve impulse normally causes secretion of one small quantum of NA from only one vesicle in each depolarized varicosity the calculation above suggests that the upper limit may be reached when 20 vesicles or one out of every 50 in the varicosity secrete one quantum each per pulse The number of vesicles in suitable position for secretion may thus be the ultimately rate limiting factor in NA secretion

?This work was supported by a grant from the Swedish Med cal Research Council (project B73-04X 30 7-05)
I thank Mrs Eivor Stjarne and Miss Sinikka Makkonen for technical assistance

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Acta physiol scand 1975 93 228–249
From the Department of Physiology University of Goteborg Sweden

Effects on the Ventral Spinocerebellar Tract Neurones from Deiters' Nucleus and the Medial Longitudinal Fascicle in the Cat

By

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Received 15 August 1974

Abstract

Baldissera F and W J Roberts *Effects on the ventral spinocerebellar tract neurones from Deiters nucleus and the medial longitudinal fascicle in the cat* Acta physiol scand 1975 93 228–249

Effects from the vestibulospinal tract (VST) and from fibres descending in the medial longitudinal fascicle (MLF) on the cells of origin of the ventral spinocerebellar tract (VSCT) have been studied with intracellular recording Out of 110 VSCT neurones the VST evoked monosynaptic EPSPs in 27 di or polysynaptic EPSPs in 56 and disynaptic IPSPs in 26 In 93 tested VSCT cells MLF stimulation evoked monosynaptic EPSPs in 6 monosynaptic IPSPs in 2 di or polysynaptic EPSPs in 25 and disynaptic IPSPs in 21 Convergence of monosynaptic EPSPs from VST and MLF was found in a small proportion of cells whereas the two descending pathways evoked reciprocal effects in another small group of neurones Convergence of monosynaptic EPSPs from VST or MLF and from group I afferents was also modest. In 9 VSCT neurones there was convergence of monosynaptic excitation and disynaptic inhibition from the vestibulospinal tract and the same pattern from MLF was recorded in 9 neurones The results are discussed in view of the hypothesis that VSCT neurones carry information on the interneuronal transmission in the spinal cord

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Lundberg (1971) proposed that the ventral spinocerebellar tract the VSCT carries information about transmission in inhibitory reflex pathways impinging upon motoneurones The VSCT according to this hypothesis might relay information in 3 ways One group of cells which receive collaterals both from the input to and the output from the inhibitory interneurones would act as input–output comparators for those interneurones Other cells may receive only collaterals of the input (or a fraction of the input) to the interneurones and may be utilized as a "reference" with respect to the "comparator" cells Finally a third group of VSCT neurones may receive collaterals from parts of both the excitatory and inhibitory inputs to alpha motoneurones and signal the inhibition reaching the moto-

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neurones against the excitatory inflow to these motoneurones This hypothesis was based mainly on findings regarding the many patterns of convergence onto the VSCT neurones (Lundberg and Weight 1971)

Extensive tests of one aspect of this hypothesis have recently been made (Gustafsson and Lindstrom 1970 Lindstrom and Schomburg 1973 Lindstrom 1973) These studies included a comparison of the characteristics of transmission through the interneurones which mediate Ia inhibition to some VSCT cells and to motoneurones with particular emphasis on the inhibition of these interneurones from motor axon collaterals This work virtually proves that the Ia inhibition to the two neuronal systems is mediated by common interneurones

However interneurones of spinal reflex pathways are characterized not only by the effects they receive from primary afferents but also by the convergent actions on them from a variety of descending systems (Lundberg 1966 1971 Hultborn 1972a b) Correspondingly VSCT neurones which receive collaterals from interneurones of inhibitory reflex pathways to motoneurones should be expected to respond to volleys in the descending pathways which influence these interneurones Furthermore if some of the peripheral connexions to VSCT are collateral to the input to inhibitory interneurones the comparator hypothesis would suggest that similar collateral connexions should be expected from the descending fibres reaching these interneurones Lundberg and Weight (1971) did in fact find that stimulation of fibres in the spinal cord evoked monosynaptic PSPs in many VSCT cells and suggested that they were mediated by collaterals of descending fibres to interneurones of reflex pathways We have now investigated these possibilities by stimulating supraspinal centres which have known effects upon motor pathways This paper reports the synaptic actions evoked in VSCT cells by volleys in the vestibulospinal tract and by fibres descending in the medial longitudinal fasciculus (MLF) A separate paper will deal with the effects from these fibre systems on transmission in inhibitory pathways to the VSCT (Baldissera and Roberts to be published)

Methods

Preparation Experiments were performed on 14 cats prepared under ether anaesthesia and subsequently treated with α-chloralose (40–50 mg/kg initially) Supplementary small amounts of Nembutal (10–0 mg/kg) were given during the course of the experiments The animals were paralyzed with Flaxedil and artificially ventilated Expiratory CO_2 concentration arterial blood pressure rectal temperature and the temperature of the mineral oil pool were monitored throughout

Laminectomies were performed in the lower thoracic (Th 11–Th 12) and in the lumbar (L 4–L 7) regions. At the thoracic level after removal of the dorsal columns the right half of the cord was transected at Th 11 dissected free in the caudal direction and mounted on stimulating electrodes. The dorsal quadrant on the left side was transected at Th 1 leaving the ventral quadrant intact Ventral roots L 5 L 6 and L 7 were cut Small patches were opened in the pia on the dorsolateral surface of the cord on the left side in the L 4–L 5 segments to permit microelectrode penetration towards the lateral border of the ventral horn

Muscle and cutaneous nerves of both hindlimbs (as listed in abbreviations) were dissected free and mounted on stimulating electrodes The skin flaps around the laminectomies and the exposed tissues in the limbs were sewn up to form pools which were filled with warm paraffin oil The occipital bone was removed caudal to the tentorium and the cerebellum exposed

Stimulation and recording The Deiters nucleus and the MLF were stimulated with thin tungsten electrodes (tip diameter 10–15 μm) insulated except at the tip (resistance 100–00 kΩ) which were inserted

stereotaxically into the brain stem through the intact cerebellum according to the technique described by Grillner Hongo and Lund (1970) Antidromic field potentials in Deiters nucleus were used to help localize that nucleus as well as mapping the thresholds for evoking descending volleys in the ventral quadrant of the cord Only the latter technique could be used to help localize the MLF electrode

Cathodal constant-current pulses of 0 2 ms duration and variable intensity were used for activating the two descending systems independently In some experime thresholds were determined at many sites spaced along parallel tracks in transverse or longitudinal planes and maps of the thresholds for descending effects were thereby obtained Surface ball electrodes were also applied to the thoracic cord above the level of the transections to permit activation of the supraspinal fibres descending in the ventral quadrant. Rectangular pulses (0 1 ms) were used for bipolar stimulation of the peripheral nerves and the spinal funicles at the low thoracic level Afferent volleys were recorded by a surface ball electrode located at the dorsal root entry zone in the lumbar enlargement A second ball electrode for recording the descending volleys was placed on the ventrolateral surface of the same spinal segment where the VSCT neurones were impaled The VSCT neurones studied were located mainly in the L 4–L 5 segments and were identified by antidromic activation of their axons in the contralateral cord at Th 12 and by the location of their somas on the lateral border of the ventral horn (Burke Lundberg and Weight 1971) Micropipettes filled by 2 M K-citrate (tip size 1–1 5 μm resistance 2–5 MΩ) were used for recording The recorded potentials were fed via a cathode follower amplifier designed to allow DC or pulse current injections through the recording electrode (Eide 1968) to a modified Tektronix 50 oscillograph having 2 pairs of beams with independent time bases and amplifications and to a CAT 1000 averaging computer The averaged responses could be displayed on the oscilloscope and photographed The resting potentials of the VSCT cells were continuously monitored on a DC coupled oscilloscope Recordings of the incoming and descending volleys were also fed through an AC coupled amplifier to both the oscillograph and the averager

Histological cont ol At the end of each experiment one or more small electrolytic lesions were made by passing 1 mA for 15 s through the tips of the brain stem electrodes The location of the lesions and the tracks of the stimulating electrodes were then checked histologically after each experiment

Abbreviations VSCT ventral spinocerebellar tract PSP postsynaptic potential EPSP excitatory post synaptic potential *IPSP inhibitory postsynaptic potential MLF medial longitudinal fascicle DN* Deiters nucleus Q quadriceps Add adductor femoris Grac gracilis Sart sartorius ABSm anterior biceps semimembranosus PBSt posterior biceps semitendinosus G S gastrocnemius soleus FDL flexor digitorum longus Pl plantaris Tib tibial nerve DP deep peroneal Sur sural nerve Saph saphenus nerve CoQ contralateral quadriceps CoH contralateral hamstring nerve (*i e* ABSm plus PBSt nerves) CoSur contralateral sural nerve VQ ipsilateral ventral quadrant FRA flexor reflex afferents

Results

Section I Postsynaptic Effects Evoked from Deiters Nucleus

1 *Monosynaptic EPSPs*

Monosynaptic EPSPs were evoked from the lateral vestibulospinal tract in 27 of the 110 cells tested about the same proportion that received monosynaptic EPSPs from the MLF These EPSPs have been selected as monosynaptic when their segmental latencies (*i e* the latency from the peak of the first positive deflection of the descending volley recorded from the ventrolateral surface of the same spinal segment as the VSCT neurones) were less than 1 ms The measured segmental latencies of monosynaptic EPSPs evoked in VSCT neurones from Deiters nucleus ranged from 0 4 to 0 9 ms (*cf* histogram Fig 1H)

In Fig. 1G the region from which a monosynaptic EPSP could be evoked in a VSCT cell with a low threshold is shown in a diagram drawn from histological sections Exploration was made in a transverse plane at the P7 Horsley Clarke coordinate The threshold for the EPSP was as low as 20 μA in the region of the Deiters nucleus and was more than 200 μA when stimulating beyond the borders of the nucleus both medially and laterally Records

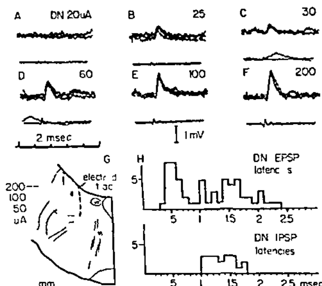


Fig 1 PSPs in VSCT neurones evoked by stimulation in the region of the Deiters nucleus The upper records in A F are superimposed traces of the potential record d intracellularly from a VSCT cell follow ing single stimuli of the indicated current intensity applied in the region of the Deiters nucleus The lower records in A F are the potentials recorded from the ventro-lateral surface of the cord adjacent to the im paled cell The diagram in G was reconstructed from transverse histological sections at the level of P 7 The dashed and dotted lines indicate the areas within which these monosynaptic EPSPs could be evoked at the ind cated stimulus strengths The ? marks in the region of Deit rs nucleus indicate zones where the threshold was less than 0 μ A The h stograms in H indicate the latencies of the PSPs (see Methods) recorded in 110 VSCT neurones

A–F of Fig 1 show the monosynaptic EPSPs recorded in this VSCT neurone with different stimulus strengths when stimulating at the site of the lowest threshold The low threshold region (100 μA) is elongated in the vertical direction and exceed the histological edges of the nucleus on the ventral side probably because of activation of vestibulospinal axons which leave the nucleus in a ventromedial direction These results are consistent with those obtained for monosynaptic vestibulospinal EPSPs in motoneurones by Grillner Hongo and Lund (1970) and indicate a vestibulospinal origin of the monosynaptic EPSPs evoked in VSCT neurones by weak electrical stimulation in this region

In another experiment in which similar mapping was performed in a transverse plane tangent to the caudal pole of the Deiters nucleus at about P8 and with the electrode in clined 30 from the vertical we found 2 distinct low threshold regions for evoking mono-synaptic EPSPs one located in the ventral part of Deiters nucleus and the other about 1 6 mm ventromedial to it (Fig 2E) This pattern suggests that the vestibulospinal effects resulted from stimulation of either the somas of cells in the caudal part of the nucleus or the axons in the vestibulospinal tract descending from neurones located more rostrally in the nucleus In the same cell a disynaptic IPSP was evoked by weak stimulation of these same regions (Fig 2B–D see below)

Often the monosynaptic EPSPs were followed by disynaptic potentials either excitatory or inhibitory For this reason, the rise times of the monosynaptic EPSPs were measurable

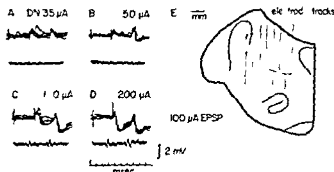


Fig. 2. EPSPs and IPSPs evoked from different sites within the vestibulospinal tract. The upper records in A D are intracellular recordings from a VSCT neurone following stimulation with the indicated strength at the site denoted ( ) in diagram E. The lower records are the descending volleys recorded from the ventrolateral cord surface The diagram in E was drawn from histological sections at about P 8 Shown are the areas from which the EPSP could be evoked with stimulus currents of 100 µA or less

only in a few cases In those cases the times to-peak were shorter than the times obtained for monosynaptic EPSPs from MLF and ranged from 0 4 to 0 55 ms this being the same range as observed for monosynaptic vestibulospinal EPSPs in extensor motoneurones (Grillner *et al* 1970) The short time to-peak indicates that the vestibulospinal volley impinging upon the VSCT neurones and motoneurones propagates along a group of fibres which are very homogeneous with respect to conduction velocity

2 Disynaptic potentials from Deiters nucleus

Stimulation in the region of the Deiters nucleus also gave rise to EPSPs and IPSPs with longer segmental latencies in VSCT cells (*cf* Fig 1H) These PSPs were believed to be evoked by polysynaptic pathways as indicated by segmental delays exceeding 1 0 ms and by their facilitation which occurred with repetitive stimulation (*cf* Grillner Hongo and Lund 1971) In addition most were facilitated by conditioning volleys from peripheral nerves (Baldissera and Roberts 1972)

Disynaptic EPSPs Disynaptic or polysynaptic EPSPs were evoked in about half (56) of the VSCT cells by vestibulospinal volleys In Fig. 3A–D are illustrated the EPSPs induced in a VSCT neurone by single (A) and repetitive (B–D) stimulation of the Deiters nucleus Its segmental latency of 1 2 ms and its potentiation after repetitive stimulation indicate that the response is mediated disynaptically The alternative possibility that the EPSP is evoked monosynaptically from slowly conducting fibres showing frequency potentiation can be excluded since the EPSP was facilitated by conditioning volleys in the contra and ipsilateral FRA (not illustrated, Baldissera and Roberts 1972)

The interneurones mediating these disynaptic EPSPs in many VSCT neurones were found to be located in the lumbar cord since EPSPs could be evoked by stimulation of the ventral quadrant of the thoracic cord (above the lesions) These EPSPs had the same segmental latencies as those evoked in the same cells by stimulation of Deiters nucleus and most probably resulted from stimulation of the thoracic vestibulospinal tract

In some cells an EPSP with a latency in the disynaptic range closely followed a mono-

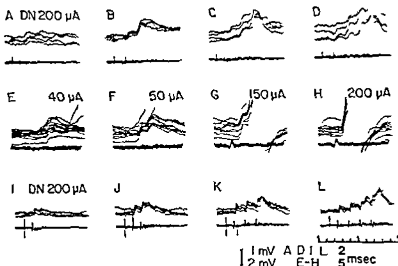


Fig. 3 Disynaptic EPSPs e oked in 3 VSCT neurones by Deiters stimulation The upper records in A D from 1 cell show the potentiating effect on the EPSP produced by repetitive stimulation The lower records are again the descending volleys Records E–H from a second VSCT neurone illustrate possible trans synapti activation of vestibulosp nal neurones (second EPSP in E–G) which becomes direct activation at 200 µA (H) Records I–L from a third VSCT neurone illustrate potentiation of a d synaptic EPSP with repet tive stimulation but no latency shift like that in H

synaptic vestibulospinal EPSP This pattern could be due to simultaneous direct and trans synaptic activation of vestibulospinal neurones (Grillner *et al* 1970) Such is likely to be the case for the cell in Fig 3E–H in which an increase of the stimulus strength from 150 to 200 µA abruptly reduces the latency of the late EPSP to the same value as that of the first one In other cells however the later EPSPs are apparently due to convergence of a spinal disynaptic excitatory path on the same VSCT neurone receiving monosynaptic ex citation from Deiters nucleus This is shown in Fig 3I–L by the strong facilitation of the late EPSP produced by repetitive stimulation and by the lack of any latency shift in the disynaptic EPSP with increasing stimulus strength

EPSPs with segmental latencies exceeding the disynaptic range were also evoked from the vestibulospinal tract In some cells these were the only response but more commonly they followed a disynaptic EPSP a combination which is probably responsible for the slow rising phase and the long duration frequently observed in the polysynaptic EPSPs from Deiters nucleus It cannot be excluded however that the late EPSPs are due to asyn chronous discharge of the interneurones in the disynaptic pathway rather than to an alter native trisynaptic path. The latency histogram of the excitatory potentials from Deiters nucleus (Fig 1H) shows a sharp peak at 0 5 ms corresponding to the monosynaptic effects and a wide distribution of the latencies between 1 0 and 2 4 ms Latencies exceeding 2 ms do not necessarily indicate the existence of separate di and trisynaptic linkages but possibly reflect differences in the excitability of the interneurones of the disynaptic path in different preparations Large latency shifts (up to 0 6 ms) are in fact observed when these interneurones are facilitated both temporally and/or spatially (Baldissera and Roberts to be published)

Disynaptic IPSPs The disynaptic IPSPs shown in the records in Fig 2A–D were evoked by stimulation of the vestibulospinal tract at the point marked X in the map (E) In that cell stimulation at the same points also evoked monosynaptic EPSPs in addition to the di synaptic IPSP Two distinct foci were found from which both PSPs could be evoked with weak stimuli (Fig 2E)

In the same neurone an IPSP was produced by low strength stimulation of the ventral quadrant of the cord (not illustrated) having the same segmental latency as from Deiters nucleus although additional earlier PSPs appeared with stronger stimulation If one as sumes that the disynaptic IPSP from the cord was mediated by the same fibres responsible for the Deiters IPSP then the latencies indicate that the interneurones are located in the lumbar cord It was possible to establish this only for a few cells in which a disynaptic IPSP was the earliest response evoked by stimulation of the ventral quadrant IPSPs from Deiters nucleus were found in 26 of the 110 VSCT neurones Their segmental latencies when measurable varied between 1 0 and 1 8 ms—the range for disynaptic connexions according to Grillner *et al* (1971) In nine cells including the one shown in Fig 2 disynaptic IPSPs closely followed monosynaptic EPSPs

In conclusion our threshold maps leave no doubt that the mono and disynaptic effects evoked in VSCT cells are mediated by the vestibulospinal tract as were the corresponding effects in motoneurones and in certain spinal interneurones (Grillner *et al* 1970 1971) In all cases low threshold foci were found within Deiters nucleus and in the region where the fibres leave this nucleus

Section II Postsynaptic effects evoked from MLF

Monosynaptic EPSPs

In about one fourth (26 out of 93) of the VSCT neurones tested a monosynaptic EPSP could be evoked by weak stimulation of the region in the brain stem which corresponds to the course of the MLF from which Grillner and Lund (1968) evoked monosynaptic EPSPs in lumbar motoneurones The EPSPs have been labeled as monosynaptic when their segmental latencies (see Methods) were less than 1 0 ms The latencies for most of these EPSPs fell between 0 5 and 0 7 ms as shown by the histogram of Fig 4K In 13 cells in which disynaptic potentials did not obscure the peak of the monosynaptic EPSP it was possible to measure the times-to peak which ranged from 0 5 to 0 8 ms

The location of the descending fibres giving this effect has been mapped in detail for 3 cells Fig. 4J shows the very restricted area (dashed line) in a transverse plane at the level of P 12 from which the monosynaptic EPSP illustrated in A–D was evoked in a VSCT neurone with stimulus currents of 20 to 100 μA This area lies completely within the histological border of the MLF A localization of the low threshold region was carried out with similar systematic tracking for another cell (dotted line) and along a single penetration for two other VSCT neurones In all the remaining cases the monosynaptic EPSPs were evoked with weak stimulation (100 μA) from points located inside the medial longitudinal fascicle as verified histologically Therefore we will assume that all of these effects were produced by the same descending system

An indication of the rostro-caudal level at which the fibres producing such monosynaptic

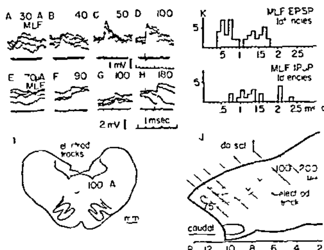


Fig. 4 PSPs evoked in VSCT neurones from the MLF The intracellular potent als (upper traces) and descending volleys (lower records polarity reversed) were recorded from 2 VSCT neurones (A–D and E–H respectively) at different stimulus strengths deli ered to the MLF area The brain stem region (P 1 level) from which these 2 monosynaptic EPSPs could be evoked with stimuli of less than 100 μA are encompassed within the dashed and dott d lines in I respectively for the 2 cells The cross hatched and the stippled areas nd cate for each cell, the zone where the threshold was lower than 0 μA The tracking diagram in J was reconstructed from histological sections in another animal made about 1 0 mm lateral to the midline The interrupted lines indicate the regions from which monosynaptic EPSPs could be evoked at the in d cated stimulus strengths in one VSCT neurone The histograms in K indicate the segmental latencies (see Methods) of the EPSPs and IPSPs evoked in 93 cells by MLF stimulation

EPSPs enter the MLF is provided in Fig 4J This map of a longitudinal plane passes dorsoventrally 1 mm lateral to the midline and indicates the threshold current necessary to evoke a monosynaptic EPSP in another VSCT cell The pattern of the threshold changes suggests that the fibres producing this EPSP enter the MLF latero-medially in the caudal medulla Since the lowest threshold point in the most caudal track was only 30 μA the fibres activated should lie within approximately 300 μm from the electrode tip at least judging from the dimensions of the hatched area in Fig 4I from which a monosynaptic EPSP was evoked with less than 30 μA Therefore it can be concluded that at this rostro-caudal position (P 12) the descending fibres approach very close to the plane of tracking 1 mm lateral to the midline

The elongated shape of the isothreshold lines indicates that either the effective fibres enter the fascicle in a somewhat tangential manner or that the distribution of the stimulating current is quite asymmetric with more current passing along the axis of the fascicle than perpendicular to it or both Since the tissue impedance of a fibre tract has been shown to be less in the axial than in the transverse direction (Ranck and BeMent 1965) the current distribution can be expected to be asymmetric However in this experiment the asymmetry is too great to be attributed to the impedance difference alone It is therefore likely that the fibres responsible for the monosynaptic EPSP enter the MLF in a tangential manner from the pontine level rather than perpendicularly from the medulla Note that a 200 μA current

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Monosynaptic EPSPs

In about one fourth (26 out of 93) of the VSCT neurones tested a monosynaptic EPSP could be evoked by weak stimulation of the region in the brain stem which corresponds to the course of the MLF from which Grillner and Lund (1968) evoked monosynaptic EPSPs in lumbar motoneurones The EPSPs have been labeled as monosynaptic when their segmental latencies (see Methods) were less than 1 0 ms The latencies for most of these EPSPs fell between 0 5 and 0 7 ms as shown by the histogram of Fig 4K In 13 cells in which disynaptic potentials did not obscure the peak of the monosynaptic EPSP it was possible to measure the times to-peak which ranged from 0 5 to 0 8 ms

The location of the descending fibres giving this effect has been mapped in detail for 3 cells Fig 4I shows the very restricted area (dashed line) in a transverse plane at the level of P 12 from which the monosynaptic EPSP illustrated in A–D was evoked in a VSCT neurone with stimulus currents of 20 to 100 μA This area lies completely within the histological border of the MLF A localization of the low threshold region was carried out with similar systematic tracking for another cell (dotted line) and along a single penetration for two other VSCT neurones In all the remaining cases the monosynaptic EPSPs were evoked with weak stimulation (100 μA) from points located inside the medial longitudinal fascicle as verified histologically Therefore we will assume that all of these effects were produced by the same descending system

An indication of the rostro-caudal level at which the fibres producing such monosynaptic

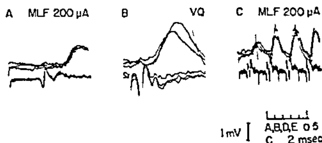


Fig 6 Disynaptic EPSPs from MLF The upper records are intracellular potentials from a VSCT neurone following single (A) or repetitive (C) stimulation of the MLF or stimulation of the ipsilateral quadrant (B) The lower records are surface recordings

cells stimulation of the ipsilateral ventral quadrant of the cord at the thoracic level produced an IPSP of about the same amplitude as the MLF IPSP with a segmental latency consistent with a monosynaptic connexion (not shown)

Disynaptic potentials from MLF

In more than half of the VSCT neurones studied repetitive stimulation of the MLF gave rise to IPSPs and EPSPs with a longer segmental delay The same criteria used by Grillner *et al* (1971) to classify PSPs from the vestibulospinal tract and MLF were used for judging these

Disynaptic EPSPs from MLF

EPSPs with segmental delays of 1 0 to 1 8 ms were recorded in 25 cells (see Fig 4K) Fig 6A–C shows presumed disynaptic EPSPs evoked in a VSCT neurone by single shocks (A) in the MLF which were facilitated by repetitive stimulation (C) In this cell the earliest potential evoked from the ventral quadrant (VQ) of the thoracic cord was also an EPSP (B) with the same segmental latency as the EPSP following the second and the third MLF shocks a finding which indicates that the effect from MLF is mediated by fast conducting axons and that the interneurones of this disynaptic pathway from the MLF are located caudal to the Th 11 In some cells EPSPs with longer delays followed monosynaptic EPSPs evoked by the same stimulation (not illustrated) These PSPs having longer latencies seemed not to be monosynaptically evoked from slowly conducting fibres since they were facilitated by repetitive stimulation whereas the early monosynaptic EPSPs were not affected by this procedure However in this case frequency potentiation in a monosynaptic pathway from slowly conducting axons cannot entirely be excluded as an alternative explanation

Disynaptic IPSPs from MLF

Repetitive stimulation of the MLF and occasionally also single shocks produced disynaptic IPSPs in 21 of the 93 VSCT cells tested One of these is illustrated in Fig 7 In this cell single shocks to the MLF (A) produced hardly any PSP however a second shock evoked an IPSP (B) which was potentiated by a third shock (C) The segmental latency of the third IPSP was 1 05 ms consistent with a disynaptic connexion

It might be that these IPSPs are evoked monosynaptically by inhibitory supraspinal neurones activated transsynaptically in the brain stem by MLF stimulation The volley

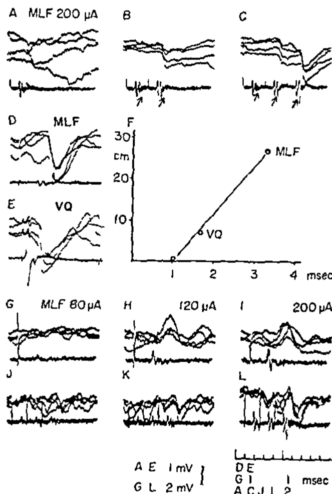


Fig 7 Disynaptic IPSPs from MLF Records A–E were taken from one VSCT neurone (upper intracellular lower surface polarity reversed) Records G–L were obtained from another cell in which MLF stimulation evoked a monosynaptic EPSP and a disynaptic IPSP Single or repetitive stimuli to the MLF were applied as indicated except for record E which illustrates the maximal response from stimulation of the ipsilateral ventral quadrant (see text) *The graph F shows the latency from the stimulus artifacts of the IPSPs for the VSCT cell in D–E as a function of the distance from the stimulus sites to the cell* For further explanation see text

in these hypothetical axons would arrive at the lumbar level later than the direct volley in the fastest *MLF* fibres and the segmental delay measured from the first positivity would thus be misleading To test this possibility one can compare the largest effect evoked in this cell from the MLF (D) with that produced by supramaximal stimulation of the ventral quadrant of the cord (E) Since an IPSP of the same size and segmental latency as the MLF IPSP was the earliest event produced by the cord stimulation it can be excluded that long descending fibres with inhibitory actions terminate directly on this VSCT neurone

If one assumes that the IPSPs from both sources were mediated by the same fibres the central latencies of these events measured from the shock artifacts to the beginning of the

inhibitory potentials can be plotted against the respective propagation distances in the graph in Fig 7F The line joining the two points intercepts the abscissa (zero distance) at about 1 ms *i e* at the segmental delay for both IPSPs This indicates that the inhibitory inter neurones are situated in the lumbar segments

Observe in this respect that the second component of the MLF volley (arrows Fig 7B C) grows in parallel with the IPSP Since the second component is much less marked when recorded from the thoracic region (not illustrated) it most probably signals the activation of a short propriospinal pathway which arises in the more rostral lumbar segments and impinges monosynaptically upon the VSCT cells This view is reinforced by the finding that disynaptic EPSPs from the MLF are also related in the same manner to the second component of the descending volley (*cf* Fig 6C) A similar comparison of the disynaptic potentials evoked from the brain stem and from the thoracic cord in several other VSCT neurones in which the first potential evoked from the cord stimulation was a disynaptic IPSP led to the same conclusion regarding the location of the interneurones

Short latency polysynaptic IPSPs were recorded in 9 VSCT cells following MLF stimula tion which also produced monosynaptic EPSPs in the same cells However in 4 neurones the latency could not be determined with sufficient accuracy to state that the neuronal linkage was disynaptic In the cell of Fig 7 G–I a single shock to the MLF evoked a mono-synaptic EPSP with a steep falling phase When passing a small depolarizing current (J–L) a disynaptic IPSP became evident which was strongly facilitated by repetitive stimulation (J–L) Note that threshold for the two effects was similar (80 μA) at this stimulation point (G J)

In most of these cells stimulation of the ventral quadrant at the thoracic level evoked the same sequence of PSPs as did the supraspinal stimulation which is also consistent with the participating interneurones being located in the caudal part of the spinal cord

Section III Convergence of Vestibulospinal and MLF Effects onto VSCT Neurones

Convergence of monosynaptic EPSPs from MLF and Deiters nucleus

In Fig 8 are shown the monosynaptic EPSPs evoked in a VSCT cell by low strength stimula tion in the MLF region (A–D) and in the lateral vestibular nucleus (E–H) The findings described below support the view that the two effects are not due to activation of the same descending fibres but are mediated by two independent systems

Results obtained previously when mapping the threshold for descending effects from Deiters nucleus and MLF as well as from the red nucleus (Baldissera Lundberg and Udo 1972) show that a 100 μA stimulus activates the nerve cells within a radius of no more than 1 mm Now both the MLF and Deiters EPSPs were maximal for stimulus strengths of less than 100 μA and the minimal distance from each electrode to the descending fibres stimulated by the other electrode was at least 2–3 mm in the most unfavourable situation (vestibulospinal fibres running parallel to the MLF at the level of the MLF electrode) Therefore coactivation of both tracts by either 100 μA stimulus is unlikely

A more conclusive argument comes from the test for occlusion between the EPSPs evoked at supramaximal strength (200 μA) from MLF (Fig 8 I) and Deiters nucleus (J) When

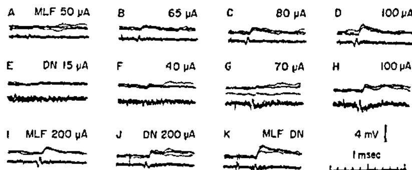


Fig 8 Convergence from Deiters nucleus and MLF onto one VSCT neurone Records A–D and I show the intracellular responses (upper) and descending volleys following MLF stimulation Records E–H and J show the responses in the same cell to Deiters stimulation Record K contains 4 superimposed responses one following Deiters stimulation alone (single trace) and 3 following stimulation of both Deiters nucleus and MLF timed to produce coincident EPSPs Note the lack of occlusion

evoked together the Deiters EPSP (single trace in Fig 8 K) and the MLF EPSP do clearly summate (upper traces in K) without any trace of occlusion The same result was obtained when increasing the strength of the Deiters stimulus up to 500 μA However when the MLF stimulation was raised to 500 μA there was apparently coactivation of the vestibulospinal fibres in transit in the medulla (not shown) In this case the MLF stimulus evoked an EPSP of the size of that in Fig 8 K which occluded the EPSP evoked from Deiters nucleus

A similar convergence of monosynaptic EPSPs evoked by low strength stimulation of both Deiters nucleus and MLF was found in a total of 6 VSCT neurones In 2 of them the EPSP from MLF and in 1 the EPSP from Deiters nucleus were followed by disynaptic IPSPs

Reciprocal effects from Deiters nucleus and MLF

Of the 20 VSCT cells with monosynaptic EPSPs from the vestibulospinal tract in which MLF stimulation was also tested 6 received disynaptic IPSPs from MLF (*i e* the same convergence as to extensor motoneurones Grillner *et al* 1971)

The inverse pattern of connexions *i e* monosynaptic EPSPs from MLF and disynaptic IPSPs from Deiters nucleus *typical for flexor motoneurones* (Grillner *et al* 1971) was found in only 2 out of 18 neurones with monosynaptic EPSPs from MLF The majority of these last cells (10 of 18) received di or polysynaptic excitation from the vestibulospinal tract

Section II Convergence of peripheral and descending effects onto VSCT cells

The pattern of synaptic connexions from primary afferents onto VSCT neurones is very complex with many different combinations of afferent inputs impinging on individual cells as described by Lundberg and Weight (1971) These authors classified VSCT neurones according to their connexions from group I muscle afferents and described some patterns

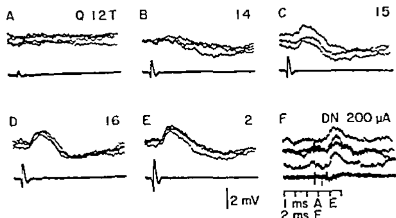


Fig. 9 Convergent effects from Deiters nucleus and Ia afferents The intracellular traces in A–E (upper) show the monosynaptic EPSP and d synapt c IPSP in response to group I a afferents in the quadriceps nerve (strength indicated as times group I threshold) Note that the EPSP is saturated at 1 5 T e before the Ib component de elops in the afferent volley The lower traces in A E are the cord dorsum potentials recorded at L 5 F shows the monosynaptic EPSP evoked from Deiters nucleus in the same cell and the accompanying descending volley

of convergence from these afferents Some rather consistent relations also emerge when the descending effects from Deiters nucleus and the MLF are compared to the peripheral input to the same cell

VSCT neurones receiving monosynaptic EPSPs from group I afferents

In 75 cells in which the peripheral input has been extensively investigated we found 33 VSCT neurones receiving monosynaptic EPSPs from group I afferents Subdivision be tween Ia and Ib effects was based on the separation of the afferent volley into Ia and Ib components (Bradley and Eccles 1953 Eccles Eccles and Lundberg 1957) When no clear separation was evident we considered as Ia those EPSPs which were evoked at threshold and were completely saturated at 1 5–1 6 T Other effects were classified as group I

Ia According to the above criteria we found 18 cells receiving monosynaptic afferent EPSPs only from Ia fibres 12 from Q and 6 from Grac and Sart In none of the cells with a Ia EPSP from Q was there convergence of monosynaptic Ia excitation from other nerves except 1 cell which received a smaller Ia contribution from Add while most of the cells with the largest Ia EPSPs from Grac and Sart received Ia EPSPs also from other nerves In all 12 Q cells stimulation of the vestibulospinal tract evoked EPSPs 9 of these EPSPs appeared to be disynaptic (1–2 ms segmental latency) while the remaining 3 were monosynaptic (1 ms segmental latency) It is of interest that 1 of these cells received both Ia monosynaptic excitation and disynaptic inhibition from Q (see Fig 9) MLF stimulation was tested in 4 of these neurones and gave disynaptic EPSP in 2 monosynaptic IPSP in 1 and disynaptic IPSP in the last one Vestibulospinal stimulation was tested only in 3 of the cells having monosynaptic EPSPs from Grac and Sart Disynaptic excitation was produced in one cell and no effect in the remaining 2 neurones MLF tested in one of these cells evoked a monosynaptic EPSP followed by disynaptic IPSP

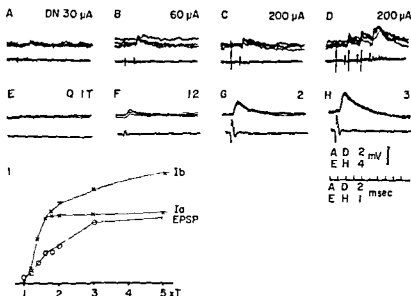


Fig 10 Convergent effects from Deiters nucleus and Ia and Ib afferents Intracellular recordings (upper records) from one VSCT neurone show d monosynaptic EPSPs from Deiters nucleus (A D) and monosynaptic EPSPs from both Ia affer nts (E F) and Ib afferents (G H) in the quadriceps nerve The graph in I shows the changes in the amplitudes of the Ia and Ib afferent volleys and the amplitude of the resulting EPSP as functions of the stimulus strengths (times Ia threshold)

Ia + Ib In 1 cell a monosynaptic EPSP from Q appeared at the Ia threshold and grew continuously over the whole range of group I activation (see Fig 10 E–H) suggesting a convergence of both Ia and Ib monosynaptic excitation from the same nerve In this cell vestibulospinal tract stimulation gave a monosynaptic EPSP (Fig 10 A–D) while MLF stimulation produced a disynaptic EPSP (not shown) Monosynaptic excitatory convergence from Ia and Ib afferents in ABSm and PBSt was found in 3 cells in our study and others in previous reports (Lundberg and Weight 1971 Lindstrom and Schomburg 1973) Stimulation of the vestibulospinal tract was tested in 2 of these 3 cells and gave a disynaptic EPSP These 3 neurones received from MLF respectively a monosynaptic EPSP a disynaptic EPSP and a disynaptic IPSP

Ib Convergence of monosynaptic excitation from Ib afferents was found to dominate in 6 cells in 3 the largest EPSP was from Q in 2 from Grac and in 1 from ABSm These neurones have been grouped together with 5 cells in which a monosynaptic EPSP was evoked in the highest range of group I effects from a single muscle nerve Out of these 11 cells 6 received di or polysynaptic excitation from the vestibulospinal tract while di or trisynaptic inhibition was found in 3 cells and mixed effects in the remaining 2 neurones. MLF stimulation was tested in 9 of these 11 cells and found to produce di or polysynaptic EPSPs in 3 di or polysynaptic IPSPs in 2 and no clear effect in 5 cells

In conclusion out of 30 cells with group I monosynaptic EPSPs convergence of monosynaptic EPSPs from the vestibulospinal tract was found only in 4 cells 3 of these cells received pure Ia excitation from Q In one of these neurones there was also a Ia disynaptic IPSP from the same nerve The fourth neurone received also monosynaptic EPSPs from Q

probably from both Ia and Ib afferents Monosynaptic EPSPs from MLF were recorded in the more limited sample of 17 of these 32 cells only in 2 neurones which received respectively group Ia EPSPs from Sart and Grac or group Ia + Ib EPSPs from both PBSt and ABSm

VSCT neurones without group I monosynaptic connexions

In 42 VSCT neurones stimulation of the dissected hindlimb nerves did not evoke any group I monosynaptic EPSPs Of these cells 22 received group I disynaptic inhibition mainly from Ib afferents

In 19 cells without any group I connexion either excitatory or inhibitory vestibulospinal tract stimulation was tested and found to produce monosynaptic EPSPs in 4 di or polysynaptic EPSPs in 10 disynaptic IPSPs in 3 and no clear effect in 2 The same test was performed in 21 of the 22 VSCT neurones with group I disynaptic IPSPs and gave monosynaptic EPSPs in 3 di or polysynaptic EPSPs in 8 or polysynaptic IPSPs in 7 and no clear effect in 3 neurones

MLF stimulation was tested in 17 neurones lacking group I connexions In 7 cells it gave monosynaptic EPSPs in 4 di or polysynaptic IPSPs while no MLF response was seen in the remaining 6 neurones Out of 19 cells with group I disynaptic IPSPs MLF stimulation produced monosynaptic EPSPs in 9 disynaptic IPSPs in 1 and no effect in 4

In summary in the group of VSCT neurones which did not receive monosynaptic excitatory connexion from group I afferents 7 out of 40 received a monosynaptic EPSP from the vestibulospinal tract and 12 out of 35 a monosynaptic EPSP from MLF

Discussion

The results presented above indicate that the cells of origin of the ventral spinocerebellar tract are subject to supraspinal inputs of great complexity which are organized in a pattern analogous to that described for certain spinal interneurones interposed in reflex pathways from primary afferents (Lundberg 1964 Hultborn 1972 a) The present results thus are in accordance with the idea (Lundberg 1971 Arshavsky *et al* 1972 Lindstrom 1973) that the VSCT mediates information regarding interneuronal transmission in the spinal cord Further support derived from an investigation of the descending control of transmission in inhibitory pathways to the VSCT will be forthcoming (Baldissera and Roberts in preparation)

The synaptic effects acting on VSCT cells from both the vestibulospinal tract and the MLF are so complex that the present results do not appear to provide the critical test of the proposed neuronal circuits (*cf* Introduction) In part this is due to insufficient knowledge of the segmental terminations of these two descending pathways This is particularly true with respect to the MLF which has not been studied with respect to its connexions onto the interneurones of segmental reflex pathways Most of our discussion will therefore be devoted to vestibulospinal effects since there exists some knowledge regarding the monosynaptic actions on the last order interneurones of reflex pathways to mononeurones from this tract (Grillner Hongo and Lund 1966 Hultborn 1972 Grillner and Hongo 1972 Bruggencate and Lundberg 1974) Furthermore most of the discussion will be focused

specifically on the monosynaptic EPSPs and the disynaptic IPSPs which are of immediate interest in relation to the comparator hypothesis

Previous classifications of VSCT cells based upon their monosynaptic Ia and Ib muscle afferent connexions (Lundberg and Weight 1971) must now be extended or revised to include the organization of descending influences from the vestibulospinal tract and the MLF The present analysis shows that the convergence of monosynaptic EPSPs from these four systems is relatively modest In our material convergence from Ia and Ib afferents was found in only 4 cells Convergence from the vestibulospinal tract and MLF was found in 6 cells from MLF and Ia or Ib in 2 cells and from Deiters nucleus and Ia or Ib in 4 cells Monosynaptic EPSPs are also evoked in some VSCT cells from the rubrospinal tract (Baldissera and Bruggencate 1969) but no attempt has been made to study the convergence from rubrospinal vestibulospinal and MLF fibres onto the same cells

Vestibulospinal effects

The input–output comparator hypothesis (Lundberg 1971 Lindstrom 1973) postulates that monosynaptic EPSPs in some VSCT neurones are mediated by collaterals from fibres which monosynaptically excite inhibitory interneurones projecting directly onto motoneurones and that disynaptic IPSPs are mediated by collaterals of these same inhibitory interneurones (*cf* Fig 11 D–F) Convergence of monosynaptic EPSPs and disynaptic IPSPs in VSCT neurones has previously been found from Ia (Lundberg and Weight 1971) and Ib afferents (Lindstrom and Schomburg 1974) Since the last order inhibitory interneurones of some reflex pathways are monosynaptically excited from the vestibulospinal tract (Grillner and Hongo 1972 Bruggencate and Lundberg 1974) it is of considerable interest that convergence of opposite effects is commonly evoked in VSCT cells from the vestibulospinal tract Although the latter results conform with the input–output comparator hypothesis it must be emphasized that it is much more difficult to interpret the significance of the vestibulospinal effects on VSCT cells than the corresponding effects from Ia afferents The reason is that while all existing information indicates that Ia afferents produce disynaptic IPSPs in motoneurones through a single interneuronal mechanism (see Hultborn 1972 *b*) there is clear evidence that this is not the case for vestibulospinal fibres These fibres inhibit flexor motoneurones disynaptically both via Ia inhibitory interneurones (Grillner Hongo and Lund 1966 Hultborn 1972) and via last order interneurones of a crossed reflex pathway from the FRA which is not shared with Ia afferents (Bruggencate and Lundberg 1974) and possibly also via a reflex pathway bilaterally activated from the FRA (Baldissera and Roberts to be published) Clearly the interpretation of the vestibulospinal effects becomes more difficult if they may be associated with a variety of segmental inhibitory pathways to motoneurones

Lundberg (1971) and Lindstrom (1973) discussed the monosynaptic EPSPs evoked in VSCT cells from ventral descending fibres mainly in relation to the monosynaptic connexion that such ventral fibres have with Ia inhibitory interneurones which in many ways have served as a model in the presentation and testing of the new VSCT hypothesis If the vestibulospinal effects on VSCT cells were related mainly to the vestibulospinal connexions with the Ia inhibitory interneurones then it might be expected that they would be frequently

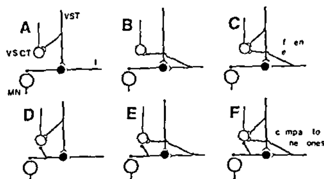


Fig 11 See text

encountered in VSCT cells with monosynaptic EPSPs and/or disynaptic IPSPs from Ia afferents We are now in the position to discuss this question on the basis of our correlation between convergent effects from vestibulospinal fibres and primary afferents onto individual VSCT cells

As mentioned above a convergence of monosynaptic EPSPs from both vestibulospinal fibres and Ia afferents as illustrated in Fig 11 C F was observed only in 4 cells The absence of Ia EPSPs in the majority of VSCT neurones receiving vestibulospinal monosynaptic EPSPs does not exclude the possibility that the vestibulospinal EPSP in VSCT cells may be mediated by collaterals from the same descending axons which terminate on Ia inhibitory interneurones because individual VSCT cells may receive collaterals only from a fraction of the input of Ia inhibitory interneurones (Lundberg 1971 Lindstrom 1973) and different cells may receive collateral representation from either Ia afferents or from the vestibulospinal fibres terminating on Ia inhibitory interneurones as in Fig 11 A B D E

According to the fractionation hypothesis (Lundberg 1971) some comparator type VSCT cells might measure activity in the Ia inhibitory pathway against collateral vestibulospinal excitation of those interneurones (*cf* Fig 11 D) However the combination of a monosynaptic vestibulospinal EPSP and a disynaptic Ia IPSP was found in only one cell Because there is now much evidence for a high individuality of VSCT cells (Lundberg and Weight 1971 Lindstrom and Schomburg 1973) significant information may be conveyed by a relatively small group of cells Thus the rare combination of vestibulospinal monosynaptic EPSP and disynaptic Ia IPSP may represent a functionally meaningful fraction of the VSCT population

Finally it should also be reemphasized that a monosynaptic vestibulospinal EPSP in some VSCT cells may be collateral to the input onto Ia inhibitory interneurones even if Ia IPSPs are not evoked in the same cells Some reference cells may signal the degree of excitation impinging upon the inhibitory interneurones but be without the collaterals from these inhibitory interneurones (*cf* Fig 11 A C) and thus be complementary to the cells with both connexions (*cf* Introduction Fig 11 D–F) Such cells should receive monosynaptic vestibulospinal EPSPs and monosynaptic Ia EPSPs as was in fact found in 3 cells Nevertheless the frequent occurrence of monosynaptic vestibulospinal EPSPs in VSCT cells not receiving Ia PSPs rather suggests that in many cells the mono-EPSPs are mediated by collaterals of vestibulospinal fibres which connect with last order inhibitory interneurone

reflex pathways other than the Ia inhibitory one—for example the crossed inhibitory private (e g not shared with Ia afferents) pathway from the FRA to flexor motoneurones (Bruggencate and Lundberg 1974) This suggestion is supported by the finding that the disynaptic vestibulospinal IPSP in many VSCT neurones is transmitted via last order interneurones of a polysynaptic pathway from the contralateral FRA (Baldissera and Roberts 1972) Thus some of the VSCT neurones receiving both excitation and inhibition from the vestibulospinal tract may serve as input-output comparators from last order interneurones of the polysynaptic path transmitting reciprocal inhibition in the crossed extensor reflex

Lundberg (1971) pointed out that it would be precipitate to suggest that all VSCT neurones are concerned with input-output comparison from inhibitory interneurones As an additional hypothesis he suggested that some VSCT cells might compare collateral effects from certain functionally related excitatory and inhibitory pathways converging onto motoneurones This hypothesis which was proposed for the VSCT cells with reciprocal Ia connexions *i e* monosynaptic EPSPs from one muscle and disynaptic IPSPs from its antagonist might be extended to the VSCT cells receiving mono or disynaptic excitation from the vestibulospinal tract and disynaptic inhibition from the MLF *i e* the pattern found in extensor motoneurones (Grillner *et al* 1971) and to those cells with the opposite connexion excitation from MLF and inhibition from Deiters nucleus as in flexor motoneurones (Grillner *et al* 1971) However Lindstrom (1973) has pointed out that the reciprocal Ia effects in VSCT cells may equally well be collateral to the input to Ia inhibitory interneurones since reciprocal Ia inhibition is found also in those interneurones (Hultborn 1972 b) Likewise it cannot be excluded that some interneurones may have analogous reciprocal connexions from the vestibulospinal tract and the MLF and that this VSCT pattern also reflects the input to certain interneurones Nevertheless we do not wish to exclude as an explanation for the observed vestibulospinal effects the possibility that VSCT cells may also receive excitatory action through collaterals of fibres impinging on motoneurones (as discussed both by Lundberg 1971 and Lindstrom 1973) In particular this arrangement might give rise to the disynaptic vestibulospinal excitation so frequently found in VSCT neurones This response is mediated by last order interneurones of FRA pathways from both ipsi and contralateral nerves (Baldissera and Roberts 1972) and might therefore be collateral to similar connexions onto motoneurones (Bruggencate and Lundberg 1974) However a more detailed discussion about the meaning of the disynaptic vestibulospinal excitation seems to be premature until more information is collected regarding vestibulospinal effects onto interneurones of reflex pathways

It should be noted that there is a potential difference between the monosynaptic actions of Ia afferents and vestibulospinal fibres The VSCT will in any case carry information regarding the Ia input to motoneurones since the same Ia afferents terminate on motoneurones and on Ia inhibitory interneurones (*cf* Mendell and Henneman 1971 Jankowska and Roberts 1972) In the case of vestibulospinal effects it is not known if the descending fibres monosynaptically connected with those Ia inhibitory interneurones projecting to flexor motoneurones are identical with the fibres having monosynaptic contact with extensor motoneurones This is but one example of the great need for caution when comparing descending effects with those evoked from primary afferents

Effects from the MLF

As mentioned above the MLF effects are even more difficult to discuss in terms of the com parator hypothesis than are the corresponding effects from Deiters nucleus The reason is that so far there is no indication that fibres in the MLF connect with the interneurones of spinal reflex pathways (Hultborn 1972 Grillner and Lund personal communication) however this problem should be investigated in more detail In a systematic investigation Baldissera and Roberts (unpublished) found that the disynaptic PSPs evoked in VSCT cells from the MLF are not influenced by hindlimb afferents Thus the pathways mediating the disynaptic PSPs seem to be private with respect to hindlimb afferents It is doubtful if there would be any need for input output information from a private descending pathway in which the interneuronal excitability is not influenced by other segmental mechanisms It might therefore be tempting to suggest that the monosynaptic and disynaptic EPSPs from the MLF are collateral to the effects on motoneurones however we wish to forward an other hypothesis Our observations (Fig. 6 and 7) indicate that MLF fibres have a synaptic relay in the upper lumbar segments If so the monosynaptic EPSPs in VSCT cells may be collateral to MLF excitation of these propriospinal neurones located in the upper lumbar segments In order to test this hypothesis it would be important to know if there is convergence from MLF and upper lumbar afferents on these presumed propriospinal neurones to motoneurones and VSCT cells If so there might be a corresponding need for ascending information from this propriospinal pathway

In a few VSCT cells stimulation in the MLF region evoked IPSPs with a segmental delay shorter than 0 7 ms in the monosynaptic range Although this effect was found in only 2 cells the finding provides another example of the highly differentiated input to individual VSCT cells Lundberg and Weight (1971) found monosynaptic IPSPs in VSCT cells upon stimulation in the lower thoracic region of ventral spinal fibres in a much higher proportion of cells (10/47) suggesting that there is another long descending inhibitory system to VSCT which may well be propriospinal although we cannot entirely exclude the possibility of another supraspinal system

The monosynaptic inhibition from MLF fibres is probably collateral to some other action in the lumbar region which is unknown since this is the first example of a monosynaptic inhibition from supraspinal centres in the lumbosacral cord Ito *et al* (1970) have shown that descending inhibitory reticular spinal neurones exist but their termination is not known Wilson Yoshida and Schor (1970) demonstrated descending monosynaptic inhibition from the medial vestibular nucleus in thoracic motoneurones but there is no evidence for corresponding effects in lumbosacral motoneurones Lundberg and Vyklicky (1966) and Jankowska *et al* (1968) have described a descending ventral pathway from the brain stem which inhibits transmission in reflex pathways at an interneuronal level but it remains to be determined whether this system acts monosynaptically on interneurones If so our findings would provide an example of collateral action to VSCT from an inhibitory input to interneurones (Lundberg 1971 Lindstrom and Schomburg 1973 Lindstrom 1973)

We wish to thank Professor Anders Lundberg for the continuous, generous support given throughout all this work We are also indebted to Dr Forrest Weight for his participation to some of the experiments.

to Mrs Rauni Larsson for excellent technical assistance to Miss Berit Nilsson for skilful typing of the manuscript and to Tomas Palm for photographic work

This work was supported by the Swedish Medical Research Council (Project No 94) Magn Bergvalls Stiftelse and the Consiglio Nazionale delle Ricerche through a N A T O fellowship to F Baldissera

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Acta physiol scand 1975 93 250-268
From the Institute of Medical Pharmacology University of Uppsala Sweden

In Vitro Uptake of Bile Acids by Choroid Plexus, Kidney Cortex and Anterior Uvea I The Iodipamide-sensitive Transport Systems in the Rabbit

By

ERNST H BÁRÁNY

Received 21 August 1974

Abstract

BÁRÁNY E H *In vitro uptake of bile acids by choroid plexus kidney cortex and anterior uvea I The iodipamide sensitive transport systems in the rabbit* Acta physiol scand 1975 93 250-268

Renal cortex anterior uvea lateral choroid plexus and terminal ileum accumulate ^{14}C-cholate glycocholate deoxycholate and chenodeoxycholate to considerable tissue/medium ratios Iodipamide partly inhibits accumulation by kidney uvea and plexus but not ileum In renal cortex the sensitive part is ~10 60 and 90 for dihydroxy acids cholate and glycocholate respectively Hippurate depresses uptake in kidney and uvea but hardly in plexus Simultaneous uptake by renal cortex and uvea of ^{14}C-cholate or glycocholate ^{131}I iodipamide and ^{125}I o iodohippurate was stud ed with unlabelled iodipamide and hippurate as inhibitors The concentration-dependence of the inhibition required the assumption of 4 partly overlapping iodipamide sensitive transport systems handling the 4 test substances the hippurate (H) system one moderately (L) and one very hippurate resistant (L_2) part of the liverlike L system and a fourth system called BS more evenly inhibitable by iodipamide and hippurate than the others The L_2-system carrie iodipamide but very little bile acids No iodipamide sensitive system clearly specialized f r bile acid transport was found The systems have only moderate affinity for bile acids and probably treat them just a large organic anions A new mathematical procedure to test the degree of complexity of composite transp rt systems without kinetic assumptions was used

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It is usual to speak about the organic anion transporting system of the kidney choroid plexus and ciliary processes This system however is composite

It has been reported that slices of renal cortex of rabbits but not of dogs accumulate the cholangiographic agent iodipamide (Biligrafin® Cholografin®) a dicarboxylic acid *in vitro* (Berndt and Mudge 1968)

The dog but not the rabbit turned out to be the exception in a variety of other species renal cortex choroid plexus and anterior uvea (or ciliary processes) accumulate the substance Even in the dog the choroid plexus has a considerable uptake while the uptake by eye or renal cortex is smaller than in other species tested Uptake is also rather small in liver slices of all species tested (Bárány 1972)

The accumulation of iodipamide by extrahepatic tissue takes place by several transport systems which can be distinguished by the differences in their relative affinities to hippurate The most hippurate sensitive one is the same as transports o-iodohippurate it may be a single system and has been called the H system It has affinity to a large variety of organic acids known to be excreted into the urine The less hippurate sensitive one has been called the liver like or L system it is liver like in having affinity to a large variety of liver excreted anions (Barány 1973 a b) While part of it is unsaturated even in the presence of 30 mM hippurate it seems to have a component with more affinity to hippurate

It is of course quite possible that the H and L-systems are not the only wide specificity transport systems for organic anions in the tissues investigated there may be such systems which accumulate neither iodipamide nor o iodohippurate

Bile and bile acids *in vitro* inhibit both the H and the L systems but are relatively selective inhibitors of the L system in the three species tested so far rabbit rat and cat (Bárány 1973 a 1974 a) This made it conceivable that bile acids or other acid steroid derivatives are the physiological substrate for at least one part of the L system The present study of bile acid uptake *in vitro* was therefore undertaken using tissues that are known to accumulate iodipamide but also terminal ileum which is known to transport bile acids actively (Lack and Weiner 1961 1966) The results show that in kidney uvea and plexus there are besides the H and L-systems several others with ability to accumulate bile acids Whether any of these is another wide-specificity anion system or maybe specialized for bile acids or other steroid derivatives is not yet clear

In the following besides several ^{14}C bile acids ^{125}I iodipamide and ^{131}I-o-iodohippurate (Hippuran®) were used as test substances The nonradioactive form of iodipamide and the non iodinated non radioactive hippurate anion were used as inhibitors To avoid confusion the labelled compounds will be called iodipamide and iodohippurate while the inhibitors will be called Biligrafin and benzoylglycine

Method

Albino rabbits of both sexes weighing about 2 kg were used Kidney eye and choroid plexus were removed and treated as described (Bárány 1974 a) The last few cm of ileum were sometimes also taken cut open and used in 15– 0 mg amounts without separation of different layers

The incubation was exactly as described Incubation bottles contained 5 ml medium with test compound and inhibitor (or only test compound) 2 slices of outer renal cortex sometimes pieces of terminal ileum 1–2 pieces of anterior uvea and sometimes ½ a lateral choroid plexus all tissues from the same rabbit Control bottles as a rule 2–4 per animal were shaken at 37 C and 1–2 at 0 C The tissues were dropped into the complete and thermostatted media and removed after exactly 0 min of incubation They were then blotted quickly weighed put singly into individual scintillation vials and prepared for counting Tissue medium ratios are always based on wet weight. In statistical calculations the animal not the slice was the statistical unit.

Counting of ^{14}C together with ^{125}I and ^{131}I in the tissue samples was done after digestion with Soluene® and dilution with a toluene based scintillator Glass vials were used to make it possible to check completeness of digestion It was found that if the glass vials were preselected for equal weight (±0 3 g) the absorption of the γ-emission from the iodine isotopes did not differ significantly between bottles Thus the vials were first counted in a γ-spectrometer and then in a liquid scintillation spectrometer Control vials with single isotopes for estimation of cross talk were always run Correction for quenching was never necessary One aimed at a ^{14}C-count about 10 times that generated in the ^{14}C-channel of the liquid scintillation spectro-

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Table II Rabbit tissues Uninhibited tissue/medium ratios (mean ± S E.) at 37 C and 0 C. Medium concentration of all ^{14}C bile acids 0 1 μM In other respects, see Table 1

		Cholate	Glycocholate	Deoxycholate	Chenodeoxycholate
Kidney cortex	Warm	15 9 ±1[illegible] (14)	17 5 ± [illegible] 0 (4)	29 9 ± [illegible] 2 (6)	37 6 ±2 3 (6)
	Cold	3 26±0 15 (15)	4 6[illegible]±0 [illegible]7 (6)	7 43±0 [illegible]9 (6)	9 7 ±0 38 (5)
Anterior uvea	Warm	7.27±0 93 (5)	5 0 ±1 4 (3)	11 1 ±1 6 (4)	15 1 ±1 8 (4)
	Cold	0 96±0 05 (5)	1 05±0 02 (3)	2 [illegible] ±0 3 (4)	3 6 ±0.2 (3)
Choroid plexus	Warm	21 [illegible] ±2 0 (5)	16 4 ±1 9 (5)	[illegible]3 3 ±3 3 (3)	21 [illegible] ±2 7 (3)
	Cold	0 77 0 80 (2)	1 5 (1)	3 2 (1)	5 5 7 3 (2)
Terminal ileum	Warm	31 8 12 2 (2)	—	22 8 13 8 ( )	3 1 15 7 ( )
	Cold	0 48 (1)	—	1.28 0 94 ([illegible])	1 90 1 44 (2)

ice-cold bottle could be used as the control non active uptake in all experiments with benzoylglycine or Biligrafin as inhibitors

Table III summarizes results with benzoylglycine (recrystallized sodium hippurate) and Biligrafin (ampoule solution) as inhibitors For each tissue the uninhibited active T/M is shown these figures are the differences between warm and cold in Table I The percent uptake in the presence of inhibition is expressed as

100 × (T/M inhibited − T/M ice-cold)/(T/M uninhibited − T/M ice-cold)

This was calculated for each experiment separately and only then averaged

There are several sets of experiments in this table The series of figures under B were derived from separate experiments run about 1 year before the rest The other figures (A) derive from 6 carefully balanced experiments with iodipamide iodohippurate and 'C cholate and 7 identical ones but with ^{14}C glycocholate the 'I and I results from these have been pooled In each such experiment one rabbit was used A few isolated figures for choroid plexus derive from other experiments

The iodohippurate and iodipamide columns agree reasonably well with previous findings (Bárány 1972) Iodohippurate uptake can be fully suppressed by benzoylglycine or Biligrafin In the iodipamide column the benzoylglycine insensitive iodipamide uptake is evident quite large in plexus (~50) smaller in renal cortex (~25) and smallest in anterior uvea (~14) But the striking finding is in the bile acid columns Part of the active bile acid uptake is quite insensitive to both benzoylglycine and Biligrafin In renal cortex close to 40° and in uvea about 30°, of cholate uptake is left even by 12 6 mM Biligrafin which depresses iodipamide and iodohippurate uptake to virtually zero For glycocholate there also is a resistant uptake but less than for cholate As Table VI shows for dihydroxy acids the resistant uptake is even higher than for cholate In the plexus the situation seems to be special Here 1 mM Biligrafin and 10 mM benzoylglycine are not approximately equivalent as in kidney and uvea Uptake of both bile acids is virtually unaffected by 10 mM benzoyl glycine while 1 mM Biligrafin leaves only 25–30 of bile acids and in two experiments at 12 6 mM leaves only 12 and 13 , cholate The transport systems of plexus seem to differ from the related ones of kidney and uvea

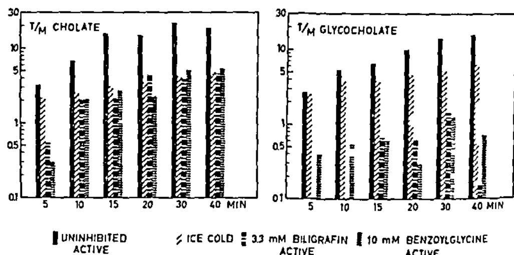


Fig 1 Time course of uptake into renal cortical slices of cholate and glycocholate Abscissae incubation time common for each group of bars Ordinates tissue/medium ratios T/M on a logarithmic scale All T/M except ice-cold are active uptake corrected for ice-cold uptake Concentrations of bile acids in medium 0 1 μM 2 slices per incubation time and medium composition

Active T/M around or below 1 0 are of course subject to large random errors The Biligrafin inhibited glycocholate values at 5 and 10 min were even negative and could not be plotted

The persistent bile acid uptake in the presence of 1 mM or more of Biligrafin shows that besides the H and L systems there is at least one other system which accumulates bile acids but has no affinity to benzoylglycine or Biligrafin We shall return to this system in a following paper

Biligrafin sensitive bile acid uptake

Is the Biligrafin sensitive part of bile acid uptake (60 % of cholate in renal cortex for instance) due to the H and L systems?

Scrutinity of Table III indicates that at least for cholate the situation must be complex In renal cortex and anterior uvea sensitive cholate uptake is identically and therefore fully depressed by 1 or 12 6 mM Biligrafin and to the same degree also by 10 mM benzoylglycine But 10 mM benzoylglycine leaves 25% of iodipamide uptake in renal cortex Evidently this highly benzoylglycine insensitive part of the iodipamide-carrying L system carries virtually no cholate Similarly the benzoylglycine insensitive part of the L system in uvea carries 14% of iodipamide but no cholate As for the choroid plexus 10 mM benzoylglycine which removes about 90 of iodohippurate uptake and about 40% of iodipamide uptake leaves bile acid uptake virtually unchanged Thus in contrast to the other tissues cholate in plexus is mainly caried by a benzoylglycine-*in*sensitive part of the transport systems

The values under A of Table I and III for renal cortex and uvea were obtained in experiments which are also illustrated in Fig 2–4 In these experiments slices of renal cortex and pieces of anterior uvea from one animal were incubated with 3 test substances one bile acid at 2 μM iodipamide at 1 3 μM and iodohippurate at 0 5 μM and with 2 10 100 and 1 000

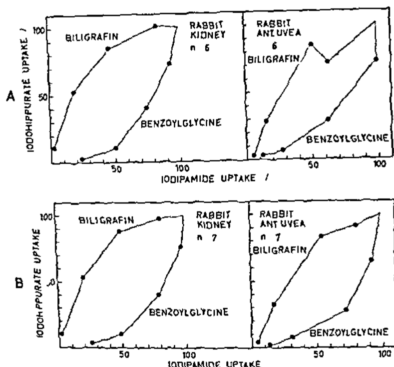


Fig 2 Relations between the uptake of iodohippurate and iodipamide with Biligrafin or benzoylglycine as inhibitors A data of cholate series B data of glycocholate series No standard error indicators plotted *in this figure* they are shown in Fig 3 and 4 which include the same data plotted differently

μM Biligrafin and 10 100 1 000 and 10 000 μM benzoylglycine as inhibitors The 4 pieces of plexus available were placed in an uninhibited and an ice cold bottle and in bottles with 1 mM Biligrafin and 10 mM benzoylglycine The figures plotted for bile acid uptake in Figs 3 and 4 were obtained as follows For each animal the percent bile acid remaining at 1 mM Biligrafin was reduced by 2% units and taken then to represent Biligrafininsensitive bile acid uptake Thus if 1 mM Biligrafin left 40 of uptake the value for insensitive uptake was taken to be 38% of uninhibited uptake (The figure 2 % was obtained in 2 extra experiments directly comparing 2 concentrations of Biligrafin) The insensitive uptake of that individual animal tissue (38% in the example) was then subtracted from *all* the uptake figures for that tissue and animal and the remaining uptake figures expressed as percent of uninhibited but sensitive or Biligrafin inhibitable uptake *For cholate in anterior uvea* the average between the value at 10 mM benzoylglycine and that estimated as just described was used as insensitive No such calculations were performed for iodipamide and iodohippurate the uptake figures plotted are percent of *total active* uptake

The results with iodipamide and iodohippurate uptake in the cholate and glycocholate experiments are shown in Fig 2 A and B The plot is as described (Bárány 1973 a) The upper branch concave towards the abscissa is with Biligrafin as inhibitor If iodohippurate and iodipamide uptake were by only one system the ellipse would be replaced by a straight

TABLE III Rabbit tissues Inhibition of uptake of 4 test substances by Biligrafin or benzoylglycine Tissue/medium ratios 'T/M are corre~ted for ice-cold uptake Percent uptake is in relation to acti e uninhibited uptake Figures marked B derive from experiments also yielding the B columns. Unmarked figures belong to the A columns The two figures with asterisks in the plexus, 12 6 mM Biligrafin row are cal~ulated using ice-cold values of Table I of the present paper and warm values from Table II of Bárány (1972) In other respects see Table I

Tissue	Inhibitor	Iodohippurate	Iodipamide
Kidney cortex	None	13 31 ± 0 39 (13) T/M	0 0 ± 1 1 (13) T/M
	1 mM Biligrafin	10 65 ± 0 46 (13)	2 1 ± 0 2 (13)
	12 6 mM Biligrafin	2 4 ± 0 4B (16) *	−0 8 ± 0 B (16) *
	10 mM benzoylglycine	3 2 ± 0 2 (13)	24 50 ± 0 76 (13)
Anterior uvea	None	6 57 ± 0 63 (13) T/M	7 87 ± 0 71 (13) T/M
	1 mM Biligrafin	4 7 ± 1 0 (13)	5 6 ± 0 7 (13) *
	12.6 mM Biligrafin	2 1 ± 1 0B (14)	0 9 ± 1 1B (14)
	10 mM benzoylglycine	4 7 ± 1 1 (13) *	13 9 ± 1 6 (13)
Choroid plexus	None	3 49 ± 0 30 (11) T/M	14 43 ± 1 01 (12) T/M
	1 mM Biligrafin	9 3 ± 3 5 (13)	5 5 ± 0 5 (13)
	3 3 mM Biligrafin	—	—
	12.6 mM Biligrafin	~10	~1 *
	10 mM benzoylglycine	9 0 ± 2 3 (13)	5? 2 ± 4 9 (13)%

diagonal line Fig 2 shows that the kidney results agree very well in the two groups of experiments as they indeed should since the nature of the tested bile acid would not affect the uptake of iodipamide or iodohippurate For uvea however there is one point out of line in Fig 2 A the cholic acid group This is due to one experiment with one point wildly off Since no reason could be found for the deviation the experiment was not discarded

Turning now to Fig 3 the cholate experiments one sees that with Biligrafin as an inhibitor the curve for cholic acid uptake by kidney cortex follows iodipamide uptake rather closely and differs markedly from the curve for iodohippurate uptake This would be explained if cholate uptake were divided between the H and the L-systems approximately in the same proportion as the uptake of iodipamide It is therefore quite surprising that with benzoylglycine as an inhibitor the bile acid curve follows the iodohippurate curve more closely than the iodipamide curve

In the cholate uvea curves of Fig 3 and the glycocholate curves of Fig 4 for kidney but not for uvea a similar but less evident trend is visible (In the uvea glycocholate follows iodipamide rather closely)

Here is something that does not seem to fit into the simple H–L-classification It looks as if at least part of the cholate is taken up by an intermediate type of system more evenly inhibitable by Biligrafin and by benzoylglycine than the contrasting H and L-systems

In order to test in a less intuitive way whether the results can indeed by explained by the presence of only the H and two L-systems with different affinities for the 4 different test substances used in these experiments two procedures were applied

First it was assumed that uptake by a slice could be represented by a sum of several uptakes following simple Michaelis-Menten kinetics and it was investigated how few

late		Glycocholate	
	B	A	B
±1 3 (6) T/M	11 3 ±0 75 (9) T/M	11 8 ±1 5 (7) T/M	10 8 ±1 4 (11) T/M
±4 1 (6)~	—	16 8 ±1 9 (7)~	—
—	39 0 ±3.2 (9)	—	10 0 ±2 3 (11)
±3 5 (6)	—	20 6 ± 4 (7)~	—
±1 0 (6) T/M	4 67±0 65 (9) T/M	5.52±0 73 (7) T/M	3 95±0 58 (11) T/M
±4.3 (6)	—	8 9±0 94 (7)	—
—	30 2 ±2 8 (9)	—	6 4 ±.4 (11)
±4 4 (6)	—	15 0 ±1 8 (7)	—
)6±0 87 (5) T/M	16 7 ±1 8 (9) T/M	13.2 ±0 97 (7) T/M	14 1 ±1 3 (11) T/M
) ±1 5 (6)~	—	27 3 ±2 4 (7)	—
—	21 (1)	—	10 (1)
—	12 13 (2)	—	—
3 ±6 8 (6)	—	103 0 ±8 6 (7)	—

Michaelis Menten hyperbolas were needed to fit the data reasonably well This is a questionable procedure for several reasons The minimum number is not necessarily the true one Also it is not certain that in slice experiments the hyperbolas are undistorted As stressed by Wedeen and Weiner (1973 1974) different layers of the slice may be exposed to markedly different concentrations because of the combination of diffusional resistance and active uptake Inhibitors may change the depth of penetration of the test substances Differences in oxygenation may also be a factor Despite these misgivings an attempt at fitting was done using a HP 9810 desk computer with a plotter and fitting visually Only the data for renal cortex were good enough for a fit even to be attempted The main emphasis was placed on fitting at the intermediate degrees of depression

First fits were produced for the iodohippurate iodipamide kidney data of Fig 2 As Table III shows the benzoylglycine resistant iodipamide uptake persists even with the uptake of iodohippurate completely depressed Evidently there is a non iodohippurate carrying L-system One such system was assumed but the combination of H and such an L system was insufficient to fit the data Another L-system with more sensitivity to benzoylglycine and carrying some iodohippurate had to be assumed too This agrees with previous findings that the L system must have one very and one moderately benzoylglycine resistant component (Bárány 1972) In Table IV the latter is called L and the highly insensitive one L_2

Table III also shows that L cannot carry any [illegible] ciable amount of cholate since cholate uptake was the same with 10 mM benzoy[illegible] ine as with 12 6 mM Piligrafin Also for glycocholate it is evident that this system car[illegible] very little Thus when attem[illegible] to fit the kidney data for cholate and glycocholate [illegible] was assumed that L_2 d d not [illegible] part The cholate data could not be fitted to only H [illegible] nd L and a new P ligrafin Sens[illegible]

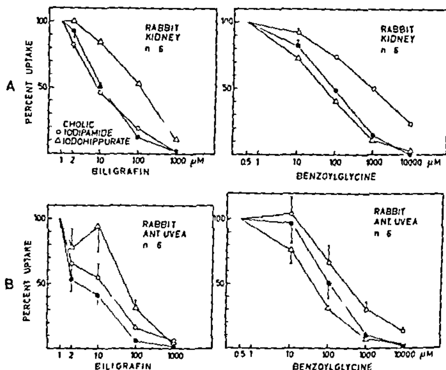


Fig. 3 Inhibition of the simultaneous uptake of cholate iodipamide and iodohippurate by Biligrafin and benzoylglycine A kidney cortex B anterior uvea from the same animals incubated together with the kidney slices. The percentage of cholic acid uptake relates to Biligrafin sensitive uptake see explanation in text Error indicators (bars) represent ± 1 S E if not visible they are hidden by the symbols

system BS had to be introduced Table IV shows that this system which is sensitive to both benzoylglycine and Biligrafin might be responsible for a considerable part of the Biligrafin sensitive bile acid uptake in kidney slices

It is evident that the constants derived from the fitting attempts and shown in Table IV are only approximations and quite uncertain Not only was the procedure unsofisticated and the fits not very precise (Table V) but the underlying assumptions as mentioned are open to doubt This last objection is the reason why instead of going on to more advanced techniques a new method was tried to test the sufficiency of only 3 uptake systems to explain the data

The objectives of the new procedure were limited It was simply a test whether uptake in the slice of the 4 test substances iodohippurate iodipamide cholate and glycocholate could be adequately represented by the sum of three independent uptake systems 1 2 and 3 without any assumptions as to the kinetics of the single system. It was necessary for this test to use the cholate and glycocholate experiments as if they had been done on the same tissue This seemed permissible since these experiments had been alternatingly done within a short span of time and in an identical manner Therefore the kidneys and eyes of the 6 animals of the cholate series and the 7 animals of the glycocholate series probably were very similar

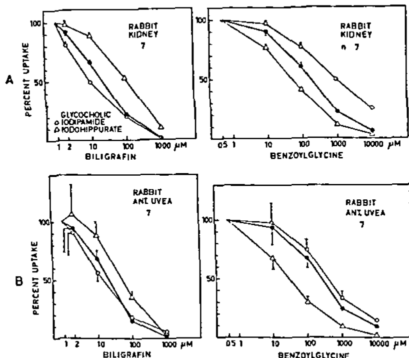


Fig 4 Same as Fig 3 but with glycocholic acid as test bile acid

The fractional contribution of the 3 systems to uninhibited uptake of *e g* test substance 1 is called C_{11}, C_{12} and C_{13}, for test substance 2 C_{21}, C_{22}, C_{23} and so on The sum of the three C for a test substance of course is 1 0 The fraction of uninhibited uptake *by the single system* in the presence of inhibitor is called x, y and z for systems 1, 2 and 3 respectively The fraction of uninhibited uptake *by the slice* in the presence of inhibitor is called a_1, a_2, a_3 and a_4 for test substance 1, 2, 3 and 4 respectively Thus the following system of equations results

$$C_{11}x + C_{12}y + C_{13}z = a_1 \quad (1)$$

$$C_{21}x + C_{22}y + C_{23}z = a_2 \quad (2)$$

$$C_{31}x + C_{32}y + C_{33}z = a_3 \quad (3)$$

$$C_{41}x + C_{42}y + C_{43}z = a_4 \quad (4)$$

where $C_{13} = 1 - C_{11} - C_{12}$, $C_{23} = 1 - C_{21} - C_{22}$, $C_{33} = 1 - C_{31} - C_{32}$, $C_{43} = 1 - C_{41} - C_{42}$

The C values are unknown but depend only on the tissue and the test substance and are independent of inhibitors and their concentrations The x y z and therefore the a values depend on the inhibitor and its concentration but only the a values are known

Now one can solve x y z out of *e g* equ (1–3) and insert the values into equ (4) One can then express one of the C values of that equation for instance C_{43} in other a and C-values only Since C values are independent of inhibitors and their concentration the value for C_{43}

TABLE IV Results of fitting 4 Michaelis Menten type systems to the renal cortex data of Fig 2–4 Explanation see text

	Test substance	Inhibitor	Transport system			
			H	L_1	L_2	BS
Fractional contribution of different systems to uninhibited uptake of different test substances	Iodohippurate (0 5 μM)		0 8	0 2	0	0
	Iodipamide (1 3 μM)		0 [illegible]	0 58	0 [illegible]	0
	Cholate (2 μM)		~0	0 35	0	0 65
	glycocholate (2 μM)		0 25	0 50	0	0 25
Affinity constants in mM^{-1} of the two inhibitors studied		*Hippurate* = benzoylglycine	[illegible]5	1 [illegible]5	0 01	25
		Biligrafin = iodipamide	5 5	100	300	90

would be independent of the kind and the concentration of the inhibitor if indeed 3 systems are sufficient to describe the situation This leads to conditions for the a values which can be tested

Straightforward calculations lead to the following expression for C_{41}

$$C_{41} = \frac{1 + k_1 R_1 + k_2 R}{k_3 + k_4 R_1}$$

which leads to

$$R_2 = (k_3 C_{41} - 1)/k_2 + R_1(k_4 C_{41} - k_1)/k_2 \qquad (5)$$

Here constants k_1 k_2 k_3 and k_4 are functions containing only C-values while $R_1 = (a_1-a_3)/(a_2-a_4)$ and $R_2 = (a_1-a\ )/(a_1-a_4)$ Now if indeed C_{41} is constant equ (5) is a straight line relation between the two ratios R_2 and R_1 both containing only observed a values This relation should be independent of the kind of the inhibitor and of the concentration One would expect different slopes and intercepts in different tissues however

The data of Fig 2–4 for kidney and uvea were tested using this relation Only values obtained with 10 or 100 μM Biligrafin and 100 or 1 000 μM benzoylglycine were utilized the others are too close to 100% or 0% Iodohippurate uptake was taken as a iodipamide as a_2 cholate as a_3 and glycocholate as a_4 The ratios R_1 and R_2 were calculated for each *animal and inhibitor concentration individually and means with standard errors computed* Fig 5 shows the result for renal cortex and uvea The points evidently do not fall on straight lines and 3 uptake systems are not sufficient to represent the experimental data Thus at least 4 systems are needed and it is interesting to note that this seems to be true also for anterior uvea which one would expect to contain fewer kinds of cells than renal cortex

The result is a limited confirmation of the results of the curvefitting attempts that 4 systems are necessary but the mathematics do not tell which test substances utilize which systems

The BS system has been assumed not to contribute to the transport of iodohippurate and iodipamide This may be wrong since it has affinity to hippurate (benzoylglycine) and iodipamide (Biligrafin) but how much its contribution is cannot be decided from the present data

TABLE V Comparison between observed values for renal cortex and those predicted by the fitted systems. The figures listed are percent uptake ± S.E. at the various inhibitor concentrations. The numbers in parentheses are number of animals, the statistical units. The bile acid percentages refer to Biligrafin sensitive only, as explained in text.

Test substance	*Inhibitor Biligrafin mM*				*Inhibitor benzoylglycine mM*			
	0.002	0.01	0.1	1	0.01	0.1	1	10
Iodohippurate								
observed (13)	99.38 ± 1.56	86.88 ± 1.45	53.15 ± 1.09	10.65 ± 0.46	75.15 ± 1.68	40.58 ± 1.06	11.06 + 0.32	3.19 ± 0.2
fitted	*95.8*	*85.8*	*53.4*	*1[illegible].5*	*83.8*	*40.6*	*12.0*	*1.8*
Iodipamide								
observed (13)	8[illegible].04 ± 1.75	48.35 ± 0.93	19.4 ± 0.59	2.08 ± 0.16	95.08 ± 1.86	76.96 ± 1.50	49.58 + 0.98	24.50 ± 0.7
fitted	81.9	53.5	18.9	3.7	95.3	79.2	48.3	24.4
Cholate								
observed (6)	93.1 ± 4.8	51.2 ± 2.6	[illegible]	2.9 ± 1.1	8[illegible].4 + 3.9	49.0 ± 1.8	13.9 ± [illegible]	0.3 ± 1.0
fitted	84.2	51.7	9.7	1.05	86.6	50.0	18.1	2.8
Glycocholate								
observed (7)	91.8 ± 3.5	65.5 ± 2.5	21.9 ± 1.[illegible]	2.3 ± 0.1	90.2 ± 3.7	60.9 ± 2.6	2[illegible].6 ± 1.1	6.9 ± 1.[illegible]
fitted	87.6	61.8	[illegible]	4.6	89.4	58.7	[illegible]4.1	3.9

Does the H system contribute to bile acid uptake? In choroid plexus it certainly does not seem to. In Table IV the zero for cholate in the H-column is a fitted value, not an assumed one as the other zeros. Thus it is only an approximation. But it seems reasonably certain that the bulk of Biligrafin sensitive cholate transport in the kidney slice is partly by a separate system called BS and partly by the somewhat benzoylglycine sensitive L_1 system. The glycine-conjugate glycocholate utilizes both these systems but also the H system. Whether all this holds also for anterior uvea is not clear. That the H system very probably plays a minor part in bile acid uptake by all three tissues agrees with the L-selectivity shown by bile acids *when used as inhibitors of iodohippurate* and *iodipamide* uptake (Barány 1974 a).

Is there any marked difference between the L_1 and BS-systems with regard to *affinity to bile acids*? Is there anything to suggest that one of the two is specifically concerned with bile acids? If this were the case, bile acids might be specially active as inhibitors for that system.

From experiments where 12 bile acids were tested as inhibitors of the Biligrafin resistant cholate uptake (2 μM ¹⁴C-cholate in the presence of 3.3 mM Biligrafin) by renal cortex (Bárány unpublished) the following acids were selected as causing less than 10% depression at 10^{-4} M: cholic acid and its conjugates and also chenodeoxycholic acid and its conjugates. Thus if at this concentration they inhibit the uptake of cholate into renal cortex in the absence of Biligrafin, at most about 4 of that inhibition (assuming 40 Biligrafin resistant uptake) is not due to an action on the Biligrafin-sensitive part.

There were 13 expts. in 10 rabbits available where the inhibitory effect of these bile acids at 10^{-4} or 10^{-5} M had been tested using 0.5 μM iodohippurate, 1 μM iodipamide and 2 μM cholate simultaneously as test substances and where cholate uptake was between 50 and 90 of controls. The data are plotted in Fig. 6 (filled symbols). The average uptake of iodohippurate was 95.4% ± 5.4, of iodipamide 71.0 ± 3.4 and of cholate 68.8% ± 2.9. The

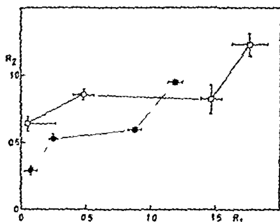


Fig 5 Test of whether 3 transport systems are sufficient to explain the data of Fig 3 and 4 If this were so each tissue would yield a straight line

Open symbols uvea filled symbols kidney cortex. Circles inhibitor benzoylglycine, squares inhibitor Biligrafin Bars indicate ± 1 S E Further explanation see text

average ratio between percent uptake of cholate and iodipamide was 0 975 ± 0 024 (n = 13) Thus the two test substances are equally depressed by the bile acids tested Now for cholate we know that ~40% Biligrafin resistant uptake was not greatly affected by the selected bile acids and the uptake figures should be corrected accordingly in order to yield values for the depression of sensitive uptake But by a coincidence the L_1 and H systems likewise seem to be responsible for about 40% of iodipamide uptake (Table IV) and they too are not greatly affected by bile acids Thus both uptake percentages would have to be corrected by nearly the same factor and the ratio cholate/iodipamide uptake would not change appreciably both would be equally depressed But iodipamide is mainly carried by the L_1 and cholate mainly by the BS system If both test substances are equally depressed both these systems also must be equally depressed Thus from these experiments none of them can be singled out as having *more* affinity for bile acids But could it be that one of them has *less* affinity for non steroidal acids or non bile acids than the other?

In the last few years several hundred acids have been tested in our laboratory as inhibitors of renal cortical uptake of iodohippurate and iodipamide Among these most are more or less H selective and depress iodohippurate uptake disproportionately while only a few (except bile acids and fusidic acid derivatives) are strongly L selective and depress iodipami uptake disproportionately Going only by the degree of H or L-selectivity a H selecti group and another the most L-selective acids all non-steroidal except fusidic acid we picked One experiment was run with each of these groups at concentrations listed in t legend of Fig 6 and cholate at 1 μM iodipamide at 2 μM and iodohippurate at 0 5 μM test substances The data are plotted in Fig 6 The contrast between H and L-selectivity shown on the left the bile acids at the concentrations used evidently are L-selective some them may even have stimulated iodohippurate uptake a little The right hand part show that for a given effect on iodipamide uptake the effect of the non bile acids on chola uptake is decidedly less than that of bile acids H-selectivity and L-selectivity made difference As mentioned the experiments with bile acids as inhibitors were done wi 2 μM cholate the other ones with 1 μM cholate The iodipamide concentration in the bi acid experiments was 1 μM in the other ones 2 μM Thus if anything, cholate uptake shoul

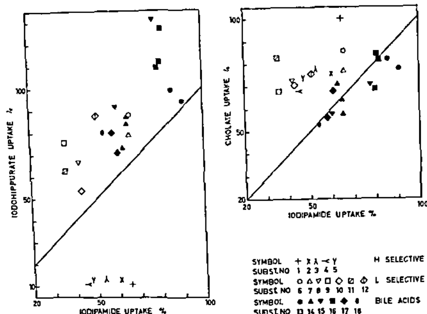


Fig 6 Comparisons of bile acids with H selective and L selective acids as inhibitors of cholate iodipamide and iodoh ppurate uptake in slices of rabbit renal cortex Explanation see text Substances and their con centrations in mM are listed below Most of the acids are carboxylic but No 7 is a disulphonated azo dye no 8 an acid tetrazole and no 11 an enolic hydrogen acid Nos 4 and 8 are anti inflammatory 6 a contrast agent 7 a food dye 10 a diuretic sulphonamide 11 an anticoagulant and 12 an antibioti

No	Acid	Conc	No	Acid	Conc
1	3 Indolacetic	1	9	Mentholglucuronide	0 3
2	6-F Nicotinic	1	10	Bumetanid	<0 01
3	Hippuric	1	11	Warfarin	0 03
4	Naproxen (Roszkowski et al 1971)	0 1	12	Fusid c	0 03
5	2 Naphthoic	0 1	13	Taurochol c	0 1
6	B l grafin	0 01	14	Chenodeoxychol c	0 01
7	Scarlet GN (Color index 14815)	0 01	15	Glycochenodeoxychol c	0 01
8	Substance 9T (Drain et al 1971)	<0 01	16	Taurochenodeoxycholic	0 1
			17	Chol c	0 1
			18	Glycocholic	0 1

be more easily and iodipamide uptake less easily depressed in the non bile acid experiments than *in those with bile acids as inhibitors* The opposite was observed

There are several possible explanations for the difference between bile acids and non bile acids The simplest one is that the BS system involved in cholate uptake but probably much less in iodipamide uptake has less affinity for non bile acids than for bile acids / possibility is that the different non bile acids have similar actions on BS and L_1 but on the H and L_r-systems which carry some iodipamide but no or little chc H selective ones may take the H system the L-selective ones a portion of L_1 ıı would cause more depression of iodipamide uptake It would be surprising substances of widely different structures were to have such a balanced action

Table VI Inhibition of uptake of 4 bile acids by Biligrafin and benzoylglycine Medium concentration of ^{14}C bile acids 0 1 μM In other respects see Table II where applicable

	Inhibitor	Cholate	Glycocholate	Deoxycholate	Chenodeoxycholate
ey	None	1 7±1 1 (14) T/M	12 8±2 0 (4) T/M	21 9±2 (6) T/M	27 9± 3 (6) T/M
ex	3 3 mM Biligrafin	41 1±5 2 (8)	6 3 (1)	88 9±7 4 (4)	84 6±5 8 (4)
	10 mM Benzoylglycine	16 5±1 8 (3)	2 4±1 2 (4)	—	—
nor	None	6 3±0 7 (5) T/M	3 9±1 4 (3) T/M	8 8±1 6 (4) T/M	11 5±1 8 (4) T/M
n	3 3 mM Biligrafin	26 6 34 7 (2)	—	104 1±14 7 (4)	74 7±6 8 (4)
	10 mM Benzoylglycine	10 4 (1)	16 8±5 6 (3)	—	—
oid	None	20 4± 0 (5) T/M	15 2±1 9 (5) T/M	19 1±3 3 (3) T/M	14 7± 7 (3) T/M
us	3 3 mM Biligrafin	15 8 (1)	8 8 (1)	60 7 (1)	67 7 (1)
	10 mM Benzoylglycine	64 3 (1)	86 4±14 0 (5)	—	—

seems possible but is not proved that the BS system has some small degree of specificity for bile acids Maybe it recognizes the steroid skeleton maybe something else It can evidently not be completely selective for bile acids or it would not be Biligrafin sensitive which is how it was picked up in the first place

The Biligrafin sensitive systems identified so far have no great affinity for bile acids Depression to 70% of gross uptake represents ~50 % inhibition of the L_1 and BS systems and this occurs at $\sim 10^{-4}$ M of cholate and taurochenodeoxycholate and at $\sim 10^{-5}$ M of chenodeoxycholate and glycochenodeoxycholate But the results presented so far have been obtained in experiments where the test substance cholate as a rule was persent at 2 μM This could have saturated a high affinity system Is there evidence for such a Biligrafin sensitive high affinity bile acid system?

It has already been indicated that the ice-cold uptake is easily saturated by bile acids Thus it might possibly be a first step in a high affinity system But since it is insensitive to Biligrafin it does not concern us here

Table VI summarizes a very limited number of experiments done with bile acids at 0 1 μM The inhibitors are the same as in Table III but in contrast to that table the experiments in Table VI did not form complete balanced series in most experiments the different inhibitors were not tested simultaneously on the same animal In some experiments only the effect of temperature and not of inhibitors was tested or vice versa The percent uptake figures with inhibitors present however all are based on uninhibited uptake ice-cold uptake and inhibited uptake measured at the same time in tissue from the same animal

A comparison of the active cholate uptake figures in different tissues between Table III with 2 μM cholate in the medium and Table VI with 0 1 μM showed that there are no significant differences The same is true for glycocholate

Nor is there any difference in the Biligrafin resistant percentage of cholate uptake In Table VI 3 3 mM Biligrafin was used in Table III the B-experiments were with 12 6 mM both concentrations virtually completely inhibit the H and L systems in kidney and uvea The Biligrafin-insensitive uptake of cholate in kidney at 2 μM was 39 % at 0 1 μM 41% Thus there is no evidence for a high affinity system at work total as well as resistant and therefore also Biligrafin sensitive uptake are all of the same order of magnitude at 0 1 as at

2 μM test substance The data for glycocholate uptake at 0 1 μM are so scanty that no conclusions can be drawn

The dihydroxy acids not only showed higher gross uptake (Table II) but also a much higher active uptake and a much higher percentage Biligrafin resistant uptake (Table VI) In fact, in some experiments Biligrafin at 3 3 mM had no effect on uptake This of course strongly speaks against a specific bile acid transport system as part of the Biligrafin sensitive group of systems

There a e discrepancies between the effect of benzoylglycine between Table VI and Table III which cannot be explained if they indeed are real The much higher benzoylglycine sensitivity apparent in Table VI cannot be due to an inhibition of the H-system in Table III by the [1] C bile acids at 2 μM which would lower its sensitivity to benzoylglycine since the bile acids are quite L-selective (Bárány 1974 a)

As a further test of the presence or absence of a high affinity system a few self inhibition experiments were run (Table VII) These experiments are not utilized in Table VI It is evident from the table that for the tested bile acids as a group no appreciable self inhibition of Biligrafin sensitive uptake occurred between 0 1 and 1 μM The main drop comes between 1 and 10 μM or higher (glycocholate) The same holds for anterior uvea (not shown) Thus if there were a high affinity system for bile acids it would have to be fully saturated already at 0 1 μM only chenodeoxycholic acid might possibly have less affinity and become fully saturated between 0 1 and 1 μM It is much more probable however that chenodeoxycholic has more affinity than the others that this causes depression already between 0 1 μM and 1 μM and that there is no really high affinity system for bile acids

Discussion

The argument of the present paper hinges on the effects of inhibitors While it is reasonable to expect that benzoylglycine (hippurate) and Biligrafin (iodipamide) act as competitors with respect to the labelled o-iodohippurate and labelled iodipamide it is not absolutely certain that their inhibition of bile acid uptake also is competitive The part of the bile acid that is transported by the H and L-systems may well be inhibited in the same manner as iodohippurate and iodipamide but what about the BS-system? Since it could be a transport system that carries bile acids but not iodohippurate or iodipamide the inhibition by Biligrafin or benzoylglycine might be of almost any kind But is there really a fourth Biligrafin sensitive system the BS-system?

The least assumptions were made in and the most reliance can be placed on the novel kind of test which showed that 3 transport systems are insufficient to explain the Biligrafin sensitive uptake of the 4 test substances (cholate glycocholate iodipamide and iodohippurate) But even this test has one questionable assumption that the 3 transport systems are independent in the sense that uptake of a test substance by the one system is not affected by inhibition of the uptake of the same substance by the others It is at least conceivable that in a slice experiment some interaction takes place in the deep layers of the slice where test substance is competed for by the different uptake systems Since the results were the same

TABLE VII Self inhibition of bile acid uptake into rabbit renal cortex All results are given as T/M Bili = 3 3 mM Biligrafin 2 individually counted slices at each concentration One kidney was first removed without killing the animal and run with 2 test substances at 37 C warm then the second kidney was removed and run at 0 C cold Note that the differences 1 2 and 3-4 which represent Biligrafin-sensitive uptake are between different animals and affected by differences between individuals The insensitivity to Biligrafin of cold bile acid uptake is evident

Test substance	Rabbit No	Inhibitor	Molarity of test substances							
			10^{-7}		10^{-4}		10^{-6}		10^{-4}	
			Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold
Cholate	1	None	20 8	-7	?1 0	2 2	14 4	1 6	9 4	1 1
	2	Bili	6 5	3 0	6 4	2 3	6 8	1 3	6 2	0 9
	1-2		14 3		14 6		7 7		3.2	
Glycocholate	1	None	-0 6	4 1	19 7	2 6	15 6	1 4	7 3	0 8
	-	Bili	4 9	3 8	4 5	-6	2 9	1 2	2.5	0 7
	1 2		15 6		15 2		12 7		4 8	
Deoxycholate	3	None	37 8	6 9	38 0	5 8	27 8	5 6	19 5	5 3
	4	Bili	25 0	6 3	25 ?	6 4	23 5	6 3	18 7	6 1
	3-4		1- 8		12 9		5 0		?6	
Chenodeoxy cholate	3	None	33 9	10 2	29 4	8 0	-3 7	6 8	-0 4	5 7
	4	Bili	30 8	9 4	29 0	8 4	24 0	7 1	18 9	5 9
	3-4		3 1		0 3		-0 3		1 4	

in uvea where the anatomy is radically different it is probable that the conclusion holds that at least 4 transport systems are indeed present but confirmatory experiments using suspensions of cells or tubules would be highly desirable

The slice technique has another limitation which becomes important where a large number of conditions such as varieties of inhibitors or their concentrations have to be tested on tissue from the same animal so that most of the available organ has to be used All slices cannot be cut strictly simultaneously they cannot be taken out of the incubation bottles and weighed strictly simultaneously and therefore the different slices have not only slightly different composition but also a slightly different history In the present experiments the time of incubation was kept rigidly constant but this implies differences in the other time factors which only hopefully were compensated by a balanced time schedule of the experiment This may matter different transport systems may be unequally sensitive to variations of this kind in some experiments where one kidney was removed from the anaesthetized animal and the second kidney one hour later Biligrafin sensitive cholate uptake was less in the second kidney than in the first despite the fact that the second kidney had been left in the living animal Evidently a suspension technique where the average composition of the tissue is identical in all respects in all the incubation bottles would be preferable Kinetic studies with such a technique might even yield information on the kinds of interaction between the different systems and their inhibitors

No evidence for a special role of the L-systems in the transport of bile acids was found From the present experiments it is evident that very little bile acids can be carried by the

highly hippurate resistant L_1 system. But in a previous paper (Bárány 1973 b) bile acids were found active as inhibitors of iodipamide uptake in the presence of 8.9–30 mM hippurate (benzoylglycine). At this concentration iodipamide uptake is mainly due to the L_2-system. Since it is improbable that this system which lacks transport capacity for bile acids has affinity for them, the reported inhibitions (mainly at 1 mM) probably were due to toxic actions (compare Bárány 1974 a).

Kidney. Transport systems for bile acids have previously been identified in the kidney. Weiner, Glasser and Lack (1964) showed reabsorptive active transport of cholic acid and its conjugates and Zins and Weiner (1968) in difficult experiments showed in addition excretory secretion of taurocholate by the proximal tubule. Both papers used the dog, which has very little of the L-system (Berndt and Mudge 1968, Bárány 1972). Taurocholate excretory secretion was inhibitable by p-aminohippurate and thus presumably utilizes the H-system while reabsorptive transport of taurocholate was insensitive to a large dose of probenecid, which inhibits both the H- and L-systems (Bárány 1972, 1973 a, 1974 b). The present paper does not deal with the Biligrafin resistant part of the bile acid uptake but it must be mentioned that probenecid at 1 mM does not affect the Biligrafin resistant uptake of cholate in kidney slices (unpublished observations). Were it not for the observation that the same is true in the anterior uvea, it would have been tempting to suggest that the Biligrafin resistant uptake system(s) is the reabsorptive one. As it is, the roles played in the renal handling of bile acids by the different transport systems are not clear.

Anterior uvea. In the uvea too it was necessary to assume a fourth Biligrafin sensitive system to explain all the data, even if the need was not as evident as in the kidney. The uvea T/M were much lower than in the other tissues and the data scatter badly. Very probably this is due to the fact that the amount of active tissue (ciliary processes) in the sector of anterior uvea used is small and not a constant fraction of total weight (compare Wålinder 1966). Whether the fourth system is the BS system is not clear.

No data seem to exist in the literature concerning bile acids in the aqueous humour in health or disease or indeed of any acid steroid metabolites. The importance of the ability of the anterior uvea to transport bile acids thus remains an open question. It could be a protective system preventing entry of blood borne acids but it could also act on locally produced material.

Choroid plexus. Transport of bile acids by choroid plexus seems not to have been observed previously. It is evident from the present experiments that at least the mix of transport systems in the choroid plexus differs from that in the uvea and kidney, maybe not only the mix but also the components differ. The scarcity of plexus tissue has made a closer analysis impossible so far.

Concluding remark. It is probable that none of the 4 Biligrafin sensitive transport systems discussed in the present paper has anything special to do with bile acid. All 4 systems may indeed be wide-specificity systems and the study may only have defined a further member of the group of organic acid transport systems.

Supported by grant 14X 733 from the Swedish Medical Research Council. I wish to thank Mrs. Inger Lideroth and Mr. Per Gerdén for excellent technical assistance.

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Acta physiol scand 1975 93 269–276
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Cerebral Blood Flow and Oxygen Consumption in the Rat in Hypoxic Hypoxia

By

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Received 9 September 1974

Abstract

JÓHANNSSON H and B K SIESJÖ *Cerebral blood flow and oxygen consumption in the rat in hypoxic hypoxia* Acta physiol scand 1975 *93* 269–276

In order to measure cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR_{O_2}) at pronounced degrees of hypoxic hypoxia the Pa_{O_2} of artificially ventilated and normocapnic rats was reduced to between 47 and 22 mm Hg for 15–25 min with subsequent measurements of CBF using a ^{133}Xenon modification of the Kety and Schmidt technique and of the arteriovenous difference in oxygen content the venous blood being obtained from the superior sagittal sinus When the Pa_{O_2} was reduced to minimal values of 22 mm Hg CBF increased 4 to 6-fold the increase in CBF being unrelated to changes in blood pressure or Pa_{CO_2} The CMR_{O_2} remained unchanged at all levels of hypoxia It is concluded that the maintenance of a normal or near normal cerebral energy state even at extreme degrees of hypoxic hypoxia depends solely on a homeostatic increase in CBF

In spite of the fact that brain cells are considered to be markedly sensitive to oxygen deficiency there are now many studies to show that the energy metabolism of the brain is extraordinarily well maintained even at pronounced degrees of hypoxic hypoxia Thus although lactic acid accumulates in the brain at Pa_{O_2} values of below about 50 mm Hg the tissue concentrations of ATP ADP and AMP remain close to normal even if the Pa_{O_2} is reduced to 20–25 mm Hg (Gurdjian *et al* 1944 Schmahl *et al* 1966 Siesjo and Nilsson 1971 Duffy *et al* 1972 MacMillan and Siesjo 1972 Bachelard *et al* 1974)

The maintenance of a normal or near normal energy state in profound hypoxia indicates that powerful homeostatic mechanisms exist that secure an almost adequate supply of oxygen to the cells One such mechanism is the increase in cerebral blood flow (CBF) which is clearly observed at Pa_{O_2} values of below 50 mm Hg and may amount to several hundred percent of normal at extreme degrees of hypoxia (Kety and Schmidt 1948 Courtice 1941 McDowall 1966 Cohen *et al* 1967 Kogure *et al* 1970) Another mechanism was recently proposed by Duffy *et al* (1972) who exposed mice to 5–3% O_2 and measured the energy consumption of the brain by means of the closed box method of Lowry *et al* (1964) Since Duffy *et al* (1972) found a decrease in energy consumption in the brain

of the hypoxic mice they postulated that the tissue can protect itself against cellular anoxia by decreasing its energy demands This attractive hypothesis receives some support from studies of transmission mechanisms in the hypoxic brain Thus hypoxia is accompanied by signs of a decreased rate of synthesis of indole and catechol neurotransmitters (Davis and Carlsson 1973 Davis *et al* 1973) Furthermore there are reports of decreased tissue concentrations of the excitatory amino acids aspartate and glutamate and of an increased concentration of the inhibitory amino acid GABA in hypoxia (Tews *et al* 1963 Wood *et al* 1968 Siesjo and Nilsson 1971 Duffy *et al* 1972 MacMillan and Siesjo 1972 Bachelard *et al* 1974)

Previous studies in man have shown that the cerebral metabolic rate for oxygen (CMR_{O_2}) remains unaltered at Pa_{O_2} values of 35–40 mm Hg (Kety and Schmidt 1948 Cohen *et al* 1967) At these degrees of hypoxia there is thus no sign of a decreased consumption of energy Since there were no previous quantitative studies of CMR_{O_2} at more pronounced degrees of hypoxia we examined CBF and CMR_{O_2} in rats after reducing Pa_{O_2} to minimal values of 22 mm Hg The objective of the study was to evaluate a possible decrease in CMR_{O_2} at degrees of hypoxia corresponding to inhalation of 5–8% oxygen A preliminary report of the findings has been published (Jóhannsson and Siesjo 1973)

Methods

The experiments were performed on male Wistar rats (3 0–4 0 g) which had free access to rat pellets and water until operation Anesthesia was induced with 2 3 halothane When unresponsive to tilting of the jar the animals were tracheotomized injected with tubocurarinechloride i p (0 5 mg kg⁻¹) and maintained artificially ventilated on 70 N O and 30 O One femoral artery was cannulated for blood pressure recording and the other for sampling of blood The latter catheter was cut to a length of about 3 cm to minimize errors due to catheter "smearing" One femoral vein was cannulated and used for infusion of fresh donor blood during the *measurement of CBF* (see Norberg and Siesjö 1974) The posterior part of the superior sagittal sinus was exposed by *means of a* small burr hole for sampling of cerebral venous blood The body temperature as measured in the rectum was kept close to 37 C by means of intermittent heating

The ventilation was adjusted to give a Pa_{CO_2} of about 35 mm Hg After a steady state period of 30 min the O concentration of the insufflated gas mixture was lowered so as to give Pao values of 20–50 mm Hg while maintaining the N O concentration at 70 In the control group the O_2 concentration was maintained at 30 throughout the experiment At Pa_{O_2} values of 0–5 mm Hg the Paco fell in spite of constant ventilation In order to maintain Pa_{CO} close to 35 mm Hg in these animals CO_2 was added to the gas mixture In the majority of the experiments the period of hypoxia was 5 min Fifteen min before the end of the hypoxic (or control) period an identical gas mixture was delivered to the ventilator from a rubber bag that also contained about 10 μC of ¹³³Xenon (obtained from AB Atomenergi Studsvik Sweden) At the end of the hypoxic (or control) period samples were taken from the artery and from the superior sagittal sinus for measurement of ¹³³Xenon activity and for total oxygen content (To_2) The bag containing ¹³³Xe was then disconnected the Xenon free gas mixture was again administered and repeated samples were taken from artery and vein for measurements of Xenon activity A new set of arterial and venous samples were taken about min after the beginning of desaturation for To_2. During the whole desaturation period fresh donor blood was infused at a rate sufficient to maintain mean arterial blood pressure above 120 mm Hg. During the 5 min hypoxic period (Pao < 35 mm Hg) there was a gradual fall of plasma pH and the arterial total O content decreased in spite of constant Pao In order to minimize the plasma acidosis six animals were made hypoxic for only 15 min (Pa 5 mm Hg or lower) and the arterial Pco was allowed to fall spontaneously by omitting addition of CO In these animals only three of which met the criteria set up for evaluation (see below) ¹³³Xenon was administered during the whole hypoxic period of 15 min.

Arterial P_O P_{CO} and pH were measured using microelectrodes operated at 37 C with due corrections for deviation in temperature T_{O_2} was measured in 25 μl samples using the method of Fabel and Lübbers (1964 see Norberg and Siesjö 1974 Borgström *et al* 1974) [133]Xenon activity in arterial and cerebral venous blood was determined with a scintillation counter (Wallac) CBF was calculated from the desaturation curves for artery and vein using the trapezoid rule (Kety and Schmidt 1948 For a more detailed account of the CBF and CMR_O methods see Eklöf *et al* 1973 Norberg and Siesjö 1974) CMR_O was calculated by multiplying CBF with the arteriovenous difference in oxygen content (AVD_{O_2}) as measured 2 min after the start of desaturation

Results

The present methods for CBF and CMR_O have now been used in several hundred determinations At normal flow values the evaluation of the arteriovenous differences in [133]Xenon activity and T_{O_2} presents no difficulties (Fig. 1 unfilled symbols) However in hypoxia the arteriovenous differences in [13] Xenon and T_O become so small that accurate determination of CBF and CMR_O is difficult The values presented here were obtained during the course of one year and represent a selection The following criteria of selection were applied (1) The blood pressure remained stable within 10–20 mm Hg during desaturation and never fall below 110 mm Hg If larger changes in blood pressure occurred the arteriovenous differences in T_O as measured before and during desaturation varied unduly (>0 5 ml $(100\ ml)^{-1}$) (2) It was possible to draw smooth lines through all [13] Xenon values for both artery and vein (an example is given in Fig 1 filled symbols) In most of the experiments that did not meet these criteria and which were therefore excluded from the material it was possible to obtain approximate CBF and CMR_O values If these experiments had been included in the material they would not have changed any of the present conclusions

Fig 2 shows that the arterial P_{O_2} but not the T_{O_2} values remained stable between the 15th and the 25th min of hypoxia The fall in T_O was obviously related to a gradual decrease in plasma pH which was observed in animals with an arterial P_O of 35 mm Hg or lower (see Fig 2) In 3 animals (not shown in the figure) Pa_O was reduced to 20–25 mm Hg for only 15 min and the Pa_{CO_2} was allowed to fall spontaneously (the values were 25 2 24 0 and 31 1 respectively) In these animals the plasma pH remained between 7 26–7 33 and the arterial T_O values were 4 35 4 59 and 4 50 ml (100 ml) respectively

The individual CBF and CMR_O values are presented in Fig. 3 There were 6 control animals (Pa_O >120 mm Hg) and 19 hypoxic animals with Pa_O values varyinge between 47 and 22 mm Hg. 9 animals had a Pa_O of 25 mm Hg or lower Of those 6 were made hypoxic for 25 min (unfilled circles) and 3 for 15 min (filled circles) The figure shows that CBF increased in all hypoxic animals At the most pronounced degrees of hypoxia (Pa_O 20–25 mm Hg) CBF increased 4–5 fold with one single value reaching 700 ml (100 g) min Although 3 of the CMR_O values at Pa_O 20–25 mm Hg (25 min of hypoxia) fell below the lower range of the control CMR_{O_2} values there was no obvious change in CMR_{O_2} at any Pa_{O_2} range

In order to allow a statistical comparison at different levels of hypoxia the material was divided into the following Pa_{O_2} ranges (1) >120 mm Hg (2) 47–37 mm Hg, (3) 33–27 mm Hg, and (4) 25–22 mm Hg The last Pa_O range was tabulated both by combining the 25 and 15 min animals (n = 9) and by choosing the 25 min animals only (n = 6) Table 1 gives the

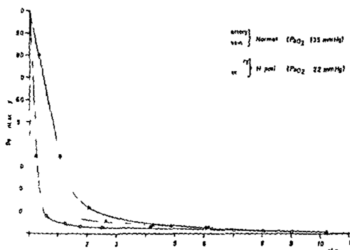


Fig 1 Representative desaturation curves for ^{133}Xenon in normoxia (unfilled symbols) and hypoxia (filled symbols) O femoral artery ◡ superior sagittal sinus

mean arterial blood pressure body temperature arterial P_O P_{CO} pH arterial and venous T_O AVD_O CBF and CMR_{O_2} Body temperature in the hypoxic groups did not vary significantly from the controls and all animals (except the 15 min animals see above) had similar P_{CO} values Mean arterial blood pressure fell significantly in the hypoxic group with Pa_O 20–25 mm Hg and at no Pa_O range was there an increase in blood pressure Thus the increase in CBF was unrelated to an increase in blood pressure Arterial T_O fell to reach 20% of normal at the most extreme degrees of hypoxia and the AVD_O was similarly reduced However there was a corresponding increase in CBF and there was thus no change in CMR_O

Discussion

As remarked in the introduction two previous studies have demonstrated that moderate degrees of hypoxia do not lead to a measurable decrease of CMR_O in man In the study of Kety and Schmidt (1948) 10% O was administered to spontaneously breathing subjects and CBF increased to 135 % of normal in spite of an associated fall in Pa_{CO} Cohen et al (1967) exposed artificially ventilated and normocapnic subjects to about 7% O_2 and recorded an increase in CBF to 170 % of normal In none of these studies did the CMR_{O_2} of the brain deviate from normal

The objective of the present study was to measure quantitatively CBF and CMR_O at more pronounced degrees of hypoxia This was achieved by reducing Pa_O to minimal values of 22–25 mm Hg in artificially ventilated rats The degree of hypoxia thus achieved is considerably more severe than in the previous studies on man This is not only due to the lower Pa_O but also to the fact that the oxygen dissociation curve of the rat (Sherwood et al 1950) is shifted to the right (P about 40 mm Hg) In the study of Kety and Schmidt (1948) inhalation of 10% O_2 which lowers Pa_O to about 40 mm Hg, reduced arterial oxygen content from 19 to 16 ml (100 ml) At a comparable reduction in Pa_{O_2} in the rat (see Table I), arterial T_{O_2} was lowered to 11 7 ml (100 ml) i e to about 55% of normal At Pa_{O_2} 22

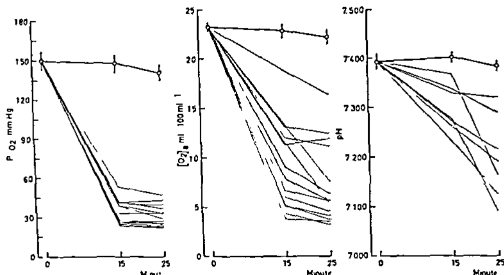


Fig 2 Individual values for Pa_{O_2}, $[O_2]_a$ and pH in the rat after 15 and 25 min of hypoxia respectively The data are compared to the corresponding values obtained in control rats (means ± S E)

25 mm Hg the arterial T_O was reduced to less than 20 % of normal in the animals that were hypoxic for 25 min Thus when compared to the previous studies of CBF and CMR_{O_2} in hypoxia the present degree of hypoxic hypoxia must be considered excessive It should be pointed out though that the severity of the hypoxic hypoxia in the present type of experiments is not only given by the reduction in Pa_{O_2} but also by the influence of plasma pH on oxygen dissociation When normocapnic animals are exposed to very low oxygen

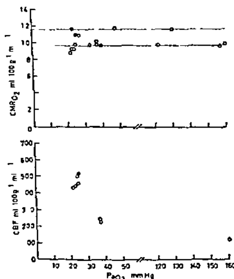


Fig 3 Individual CBF and CMR_O values in the rat after 25 min of exposure to hypoxia In 3 animals (filled circles) the period of hypoxia was 15 min and Pa_{CO_2} was allowed to fall spontaneously

TABLE I Effect of hypoxia on physiological parameters cerebral blood flow and cerebral metabolic rate in rats. Means ± S E.M

Number of animals	PaO_2 mmHg	Arterial pressure mm Hg	Temperature C	$PaCO_2$ mm Hg	pH	$[O_2]_a$ ml (100 ml)$^{-1}$	$[O_2]_v$	AVDo	CBF ml (100 g min)$^{-1}$	CMRo
6	140 ±6	143 ± [illegible]	36 8 ±0.2	37 3 ±0 1	7 382 ±0 009	[illegible]2 18 ±0 72	1[illegible]9[illegible] ±0 65	9 [illegible]5 ±0 71	114 ± 6	10 31 ±0 34
5	40 8 ±2.1	133 ± 3	36.7 ±0.2	38 4 ±1 7	7 257 ±0 061	11 74 ±1 41	7 06 ±0 99	4 68 ±0 73	235 ±21	10 33 ±0 36
4	30 5 ±1 5	1[illegible]4 ±11	37 1 ±0 1	37.5 ±1 4	7 146 ±0 0 0	5 54 ±0 39	3[illegible]5 ±0 50	[illegible] 43 ±0 14	439 ±[illegible]8	10 55 ±0 31
6	[illegible]3 5 ±0 6	1[illegible]8 ± 5	36 5 ±0 2	35 4 ±0 7	7 150 ±0 0[illegible]1	4 08 ±0 13	[illegible]06 ±0 31	2 0 ±0 06	516 ±41	10 36 ±0 71
9	23 4 ±0 5	1[illegible]7 ± 3	36.5 ±0	[illegible] 5 ±1 6	7 185 ±0 0 9	4 66 ±0 44	2.18 ±0 21	2 03 ±0 04	5 8 ±28	10 65 ±0 48

concentrations for 20–30 min an excessive plasma acidosis develops aggravating the hypoxia Furthermore at any given arterial oxygen content a fall in blood pressure adversely affects the oxygenation of the brain If spontaneously breathing animals are exposed to 5% O_2 there is a fall in Pa_{O_2} to about 20 mm Hg (LEWIS *et al* 1974) but since the animals hyperventilate vigorously most of them show a plasma alkalosis that would promote oxygenation of hemoglobin at the existing Pa_{O_2} There are thus reasons to believe that the degrees of hypoxic hypoxia studied presently is *at least* as severe as that encountered when spontaneously breathing rats are exposed to 5 % O

Available evidence indicates that the Kety and Schmidt technique (1948) as applied to the rat quantitatively measures CBF in supratentorial cerebral (cortical) regions (EKLÖF *et al* 1973 NORBERG and SIESJÖ 1974) Of particular importance are the facts that the tissues drained by the superior sagittal sinus do not contain slowly perfused tissue masses and that extracerebral contamination of venous blood can be considered insignificant (see NORBERG and SIESJÖ 1974) In view of these facts there are no *a priori* reasons to suspect that systematic errors should be expected in high flow situations like hypoxia Our data show an unchanged CMR_{O_2} at moderate degrees of hypoxia (see Table I) and in hypercapnia (EKLÖF *et al* 1973) quite in agreement with previous studies (KETY and SCHMIDT 1948 COHEN *et al* 1967) The difficulties encountered in measuring CBF and CMR_O at extreme degrees of hypoxia are therefore technical and due to the low arteriovenous differences in T_{O_2} and [133]Xenon activity Due to these difficulties conclusions should be drawn with some caution when minor differences in CMR_{O_2} between the groups are considered

The present results unequivocally show that CMR_{O_2} is maintained close to normal even at extreme degrees of hypoxic hypoxia The data cannot exclude the possibility that a minor reduction in CMR_O may occur in some animals that have Pa_O reduced to about 20 mm Hg for 25 min and that have an excessive plasma acidosis and therefore also a very marked lowering of arterial T_{O_2} However although such a reduction would be com

patible with results demonstrating a minor change in cerebral energy state at Pa_O values of about 20 mm Hg (MacMillan *et al* 1974) the present results fail to give clear proof of its existance At any rate it can be concluded that there is no measurable reduction in CMR_O before the energy state of the tissue is affected Furthermore the present techniques fail to corroborate a reduction in CMR_O of the magnitude reported by Duffy *et al* (1972) These authors calculated a fall in CMR_O, by 40% when using the rate of disappearance of glucose and glycogen and a 15% reduction when using instead the rate of increase in lactate It has subsequently been demonstrated that a reduction in body temperature of the order occurring in the experiments of Duffy *et al* (1972) (3–4 C) lowers CMR_O, by 15–20% (Hagerdal *et al* 1974) Thus the fall in body temperature can explain all of the reduction in CMR_O derived from lactate data and a large part of that calculated from the figures for glucose and glycogen We therefore conclude that any theory that attempts to explain energy homeostasis in the brain in hypoxic hypoxia must take into account that not only the energy state but also CMR_O are maintained close to normal even at extreme degrees of hypoxia

This study was supported by grants from the Swedish Med cal Research Council (Projects No 14X ?63 and 14X 2179) from the Swed sh Bank Tercentenary Fund from U S PHS Grant No 5 R01 NS07838 06 from NIH and from Magnus Bergvalls Stiftelse

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Acta physiol scand 1975 93 277–285

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Effect of Substance P on Various Vascular Beds in the Dog

By

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Received 25 September 1974

Abstract

HALLBERG D and B PERNOW *Effect of Substance P on various vascular beds in the dog* Acta physiol scand 1975 *93* 277–285

Us ng the electromagnetic flowmeter technique the blood flow in the aorta carotid hepatic superior mesenteric renal and femoral arteries and portal ein was recorded during continuous i v infusion of synthetic Substance P (SP) in 8 dogs. Systemic and portal blood pressures were recorded A significant decrease in mean arterial blood pressure was recorded at infusion of SP in the femoral vein at a rate of 2 5 ng min × kg b w ^{1} or higher Portal venous blood pressure increased A rapid increase in the carotid hepatic mesenteric and portal blood flow was obtained at infusion rates of 1 2 ng × min^{-1} kg b w^{-} or higher The femoral artery responded with a late transient increase in flow with a return to the base level wh le the infus on was still in progress The renal artery blood flow decreased slightly at low infus on rates and increased at h gher At SP infusions in the portal vein the infusion rate had to be increased to 0 ng × m n kg b w^{-} or h gher before any general vascular reactions were recorded i dicating that the liver has a h gh capacity for inactivat ng SP

In their original communication on Substance P (SP) Euler and Gaddum (1931) observed that besides contracting smooth muscle organs SP produced a transient fall in the arterial blood pressure which they attributed to a peripheral vasodilatation This observation was confirmed with partly purified SP preparations (Pernow 1953 a) but no detailed studies have so far been performed in animals in order to further elucidate the circulatory actions of SP In man close arterial infusion of SP provoked a considerable increase in skin and muscle blood flow supporting the theory that the circulatory action of SP is sited peripherally (Lofstrom, Pernow and Wahren 1965)

After the recent purification and amino acid sequence analysis (Chang and Leeman 1970 Studer Trzeciak and Lergier 1973) SP is now available in synthetic form (Tregear *et al* 1971 Fisher *et al* 1974) Its extremely high potency both as a hypotensive compound and a smooth muscle stimulating factor in the intestine justifies a detailed study of the effect of SP on various vascular beds with special reference to the splanchnic area.

LEWIS L D U PONTÉN and B K SIESJÖ Arterial acid–base changes in unanaesthetized rats in acute hypoxia *Resp Physiol* 1973 *19* 312–321

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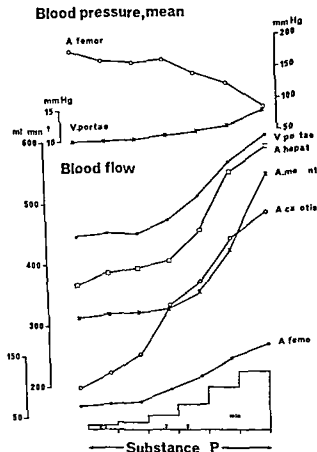


Fig 1 Effect of SP on the mean blood pressure of the femoral artery and portal vein and on the blood flow of the portal vein and the hepatic mesenteric carotid and femoral arteries SP was given as a continuous infusion in the femoral vein with a gradual increase in the infus on rate

Results

A *SP infusion in the femoral vein* (Table I Fig 1–2)

Blood pressure Before SP infusions the mean arterial blood pressure was 153 mm Hg (range 100–180) and the mean portal vein pressure 12 7 mm Hg (range 8 5–20 0) Infusions of SP in doses of 2 4 ng × min^{-1} kg b w^{-} or higher invariably caused a decrease in the mean arterial blood pressure In some dogs a hypotensive effect was obtained at infusion rates of only 0 6–0 9 ng min^{-1} kg b w^{-1}

The portal vein pressure increased in all dogs at an infusion rate of 1 2–1 8 ng min × kg b w ^{1} and in some dogs already at 0 3 ng min^{-1} kg b w^{-1}

Both pressure changes displayed an approximate dose response relationship

Blood flow Data on the basal flow in the vascular beds studied are given in Table I Even at infusion rates of only 0 3 ng min ^{1} kg b w ^{1} blood flow was occasionally increased in all the sections studied except the renal artery For the dogs as a group a significant increase in the blood flow was obtained for the carotid and hepatic arteries and in

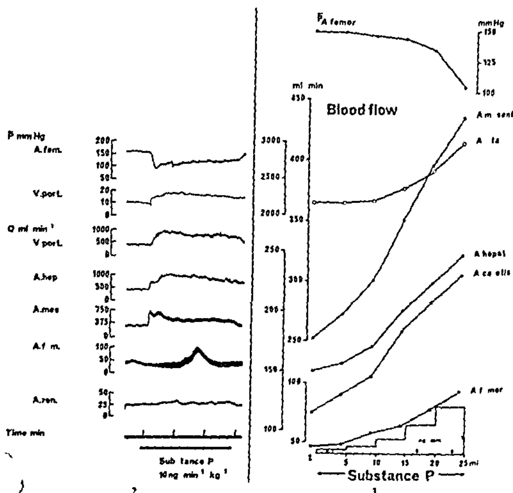


Fig. Original recording of the effect of SP infusion (10 ng min⁻¹ kg b w⁻¹) on the mean blood pressure of the femoral artery and portal vein as well as on the blood flow of the portal vein and the hepatic mesenteric femoral and renal arteries

Fig. 3 Effect of i v SP infusion at a gradually increased rate on the arterial mean pressure and blood flows in the aorta and the mesenteric hepatic carotid and femoral arteries

fusion rate of 0 6-0 9 and for the femoral and mesenteric arteries and the portal vein at 1.2-1 8 ng min kg b w Further increases in the infusion rate gave an approximate dose response relationship

In the renal artery on the other hand the blood flow decreased slightly in most dogs during infusion rates of 0 3-2 0 ng min⁻¹ kg b w⁻¹ while an increase in flow was recorded at higher infusion rates However due to large interindividual differences in the small material studies neither the decrease in flow at low infusion rates nor the increase at higher rates was significant

The flow pattern during continuous SP infusion varied greatly between the vascular beds studied In the hepatic and mesenteric arteries as well as in the portal vein flow rose rapidly

TABLE II Effect of SP on vascular resistance (of control value) Data are given as mean value ± S E

		Control pru	Substance P (ng × min × kg b w⁻¹)						
			0 3–0 5	0 6–0 9	1 2–1 8	2 4–3 6	4 8–7 2	9 6–14 4	19 ? 4 8
Carotid art	n=3	0 9±0 2	82±8	64±7	56±5	5?±5	36±7	32±6	
Renal art	n=5	0 8±0 2	104+2 ns	105±3 ns	104±2 ns	95±3 ns	94±4 ns	94±4 ns	
Femoral art.	n=8	4 2±0 1	88±2 ns	81±3 p<0 05	64±4 p<0 01	48±4 p<0 001	36±3 p<0 001	29±3 p<0 001	24±5 p<0 001
Hepatic art	n=7	0 7±0 1	100±2 ns	86±3 ns	84±3 ns	71±4 p<0 05	47±3 p<0 001	41±3 p<0 001	28±5 p<0 001
Mesenteric art.	n=7	0 7±0 2	96±3 n	95±2 n	78±4 p<0 05	61±5 p<0 01	50±5 p<0 001	34±4 p<0 001	
Portal vein	n=6	0 3±0 01	100±3 ns	9 ±3 ns	85±4 ns	79±3 ns	54±4 p<0 01	46±4 p<0 001	35±6 p<0 001

and was generally maintained at the higher level during the whole infusion period though it occasionally fell back slowly In the femoral artery in particular and to some extent in the carotid and renal arteries an early peak flow was recorded followed by a successive decrease sometimes down to the preinfusion level (Fig 2)

The aortic blood flow was recorded simultaneously with that of the carotid hepatic mesenteric and femoral arteries in 2 dogs (Fig 3) Increases in the carotid hepatic and mesenteric artery blood flow were recorded at infusion rates of only 0 3–0 7 ng × min⁻¹ × kg b w⁻¹ while the aortic flow did not increase until the rate of infusion had reached 2 4–3 6 ng min⁻¹ kg b w⁻¹ concomitant with a decrease in the mean arterial blood pressure A further gradual increase in the infusion rate successively raised the aortic flow

The effect of SP on vascular resistance is summarized in Table II showing a decrease in resistance in all vascular beds except the renal artery

The interval before the flow effects of SP appeared after the start of i v infusion varied greatly between the vascular beds (Fig 4) Regardless of the infusion rate the flow response

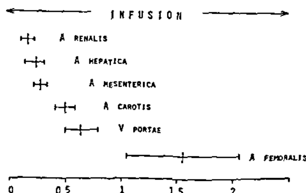


Fig. 4 Onset of blood flow reactions to i v SP infusions mean and range

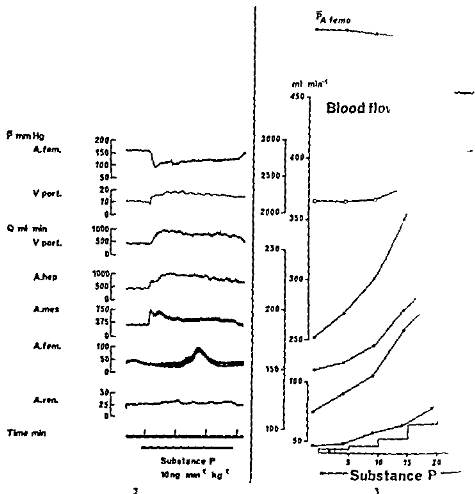


Fig. 2. Original recording of the effect of SP infusion (10 ng min⁻¹ kg b w ⁻¹) on the mea[n] [pres]sure of the femoral artery and portal vein as well as on the blood flow of the portal vein an[d] [the] mesenteric femoral and renal arteries

Fig. 3 Effect of i v SP infusion at a gradually increased rate on the arterial mean pressure a[nd] flows in the aorta and the mesenteric hepatic carotid and femoral arteries

fusion rate of 0 6–0 9 and for the femoral and mesenteric arteries and the portal vein 1.2–1 8 ng min⁻¹ kg b w Further increases in the infusion rate gave an approximate d[ose] response relationship

In the renal artery on the other hand the blood flow decreased slightly in most d[ogs] during infusion rates of 0 3 2 0 ng min⁻¹ kg b w⁻¹ while an increase in flow was recorded at higher infusion rates However due to large interindividual differences in the small material studies neither the decrease in flow at low infusion rates nor the increase at higher rates was significant

The flow pattern during continuous SP infusion varied greatly between the vascular beds studied In the hepatic and mesenteric arteries as well as in the portal vein flow rose rapidly

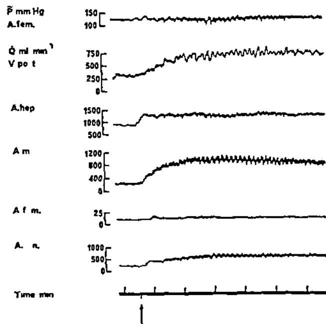


Fig. 6 Effect of glucagon on the arterial mean pressure and blood flows of the portal vein and the hepatic mesenteric femoral and renal arteries The arrow indicates when 1 mg glucagon (Eli Lilly International Corp Indiana, USA) was given as a single injection into the femoral vein

1963 Laszlo 1963 Baldauf Harnacke and Zetler 1968) and the intestine (Euler and Gaddum 1931 Pernow 1953) it is of special interest to note the considerable sensitivity of the carotid and splanchnic vascular beds to this substance Further studies will show whether this prevalence indicates a special function of SP in these areas

The few studies which have been presented so far on the pharmacological actions of chemically pure or synthetic SP suggest that the substance has a remarkably potent effect on the circulation (Pernow and Rosell 1975) as well as on intestinal motility (Fisher *et al* 1974) This is specially striking when the effect of SP on the splanchnic vascular bed is compared with that of other vasoactive and smooth muscle stimulating polypeptides which occur naturally in the gastrointestinal tract such as cholecystokinin (CCK) octapeptide of CCK (Ondetti *et al* 1970) and vasoactive intestinal peptide (VIP) (Said and Mutt 1970) Using the same technique as in this paper Thulin (1970) and Thulin and Olsson (1973 a b) obtained a fall in the systemic blood pressure and an increase in the blood flow of the hepatic and mesenteric arteries and the portal vein with CCK and synthetic octapeptide CCK at infusion rates of approximately 100 ng $min^{-1} \times kg$ b w or higher Similar effects were obtained with 30 ng VIP and higher The threshold infusion rate of SP in the present study was only 0 5–1 5 ng min^{-1} kg b w illustrating the potency of this substance Glucagon another vasoactive polypeptide elicits circulatory effects quite different from those of SP with a strong increase in the mesenteric portal and hepatic flows almost no increase in the femoral flow and no effect on arterial blood pressure (Fig. 6) A mixture of amino acids used for parenteral nutrition increased the splanchnic blood flow more than in other vascular beds The increase in peripheral flows in that study was parallel with a rise

in cardiac output No effects on the arterial blood pressure were observed (Andreen *et al* 1974)

The very large difference in the threshold infusion rate of SP in the portal as compared to the femoral vein indicates a considerable capacity of the liver to inactivate the substance Earlier studies on the inactivation of SP have shown that tissues such as brain (Gullbring 1943) intestine (Gullbring 1943 Pernow 1955) and kidney (Arvidsson Pernow and Swedin 1956) are highly potent as activators of SP whereas its inactivation in the lung is negligible (Boileau Compeau and Biron 1970) In this connection it is interesting to note that portal infusion of cholecystokinin provoked a circulatory effect which was only slightly less pronounced that that of an infusion in the caval vein (Thulin and Olsson 1973) indicating that the liver has a low capacity for inactivating CCK

This study was supported by grants from the Swedish Medical Research Council (04X-4495) and Karolinska Institutet We are indebted to Dr K. Folkers Institute for Biomedical Research the University of Texas Austin Texas USA for the generous gift of synthetic Substance P

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Acta physiol scand 1975 93 286–288
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Effects on Fluid Balance of Intraventricular Infusions of Prostaglandin E_1

By

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It was originally shown by Milton and Wendlandt (1970) that injections of minute amounts of prostaglandin E_1 (PGE_1) into the third cerebral ventricle produce hyperthermia in the cat The same response to intraventricular injections of PGE_1 and PGE_2 has been obtained subsequently in other species among them the sheep (Hales *et al* 1973) Unlike pyrogen fever the PGE induced rise in body temperature is not prevented by antipyretics (Milton and Wendlandt 1971) known to inhibit PG synthesis It has led to the suggestion that prostaglandins may be intermediaries of the fever response to pyrogen (Vane 1971) The observation that intracarotid infusions of PGE_1 inhibit the water diuresis of rats suggests that release of antidiuretic hormone (ADH) may be another centrally mediated PG-effect (Vilhardt and Hedqvist 1970) This made it of interest to study the effects of slow infusions of PGE_1 into the third ventricle of the conscious goat

Two female goats (b wt 34 and 37 kg) were used The animals were supplied with permanent platinum iridium cannulas in the third cerebral ventricle The construction of the cannula-system and the infusion technique has been described earlier (Åkerlund Andersson and Olsson 1973) The infusion rate was 10 μl/min and the duration of the infusion periods was 1 h PGE_1 was administered in either isotonic (0 15 M) or 0 45 M NaCl in amounts of 1 5 or 1 μg min Control infusions were 0 15 or 0 5 M NaCl The expts were performed at a room temperature of 22 C and the ear surface temperature of the goats was continuously recorded during the expts The rectal temperature was measured before and 5 to 10 min after the infusions The urine was collected in 10 min samples via a retention catheter for determination of osmolality and [Na] Hydration was accomplished by giving the goats 100 ml kg of 37 C water 90 min prior to the start of the intraventricular infusions A plasma osmolality value of 290 mosm kg was used for the calculation of renal free water clearance (C_{H_2O}) during hydration

13 intraventricular infusions of PGE were performed of which 10 were made in the hydrated animal The higher dosage of PGE_1 was given in 6 of the hydration expts

Temperature regulation None of the infusions of PGE into the cerebrospinal fluid (CSF) of the third ventricle induced any rise in rectal temperature As indicated by a surface temperature of 34 1 C ear vasodilatation was present at the start of the infusion in 9 expts This ear vasodilatation persisted throughout the infusion periods

Effects on fluid balance In the hydrated animal the infusion of PGE_1 in isotonic NaCl

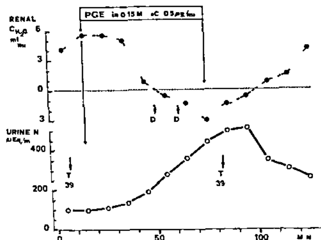


Fig. 1 Inhibition of the water diuresis, drinking, and natriuresis elicited by the infusion of prostaglandin E_1 (PGE) into the third cerebral ventricle of a hydrated goat No fever developed during the infusion At the arrows marked *Dr* the animal drank 80 and 100 ml of water C_{H_2O} = free water clearance T = rectal temperature

(n = 7) invariably caused a temporary inhibition of the water diuresis with a negative renal free water clearance (C_{H_2O}) developing in 5 of the expts In 3 of these expts the goats drank 100 to 200 ml of water during the second half of the infusion period A 4 to 6-fold increase in the renal Na^+ excretion was observed towards the end of the infusions (Fig 1) In the water replete non hydrated animal corresponding infusions of PGE_1 in isotonic NaCl (n = 3) induced the cumulative drinking of 0 7 to 1 1 of water Control infusions of 0 15 M NaCl did not affect the fluid balance of the animals

Intraventricular infusions of *PGE in 0 45 M NaCl* were made in the hydrated animal only (n = 3) These infusions caused a profound and long lasting inhibition of the water diuresis (negative C_{H_2O} for 70 to 90 min) and induced the drinking of 0 8 to 1 3 l of water A 10-fold increase in renal Na^+ excretion developed during these infusions The antidiuretic dipsogenic and natriuretic responses to control infusions of 0 5 M NaCl (n = 5) were much less pronounced

Discussion The amount of PGE administered into the CSF of the third ventricle of the goat was smaller (in relation to b wt) than that generally used in other species to induce hyperthermia Hence the lack of a thermogenic effect in the goat can not be regarded as evidence against the hypothesis that prostaglandins of the E type are intermediaries in the fever mechanism However the results of these preliminary expts indicate that PGE_1 administered into the third ventricle of the goat primarily affects other homeostatic mechanisms than the regulation of body temperature PGE_1 elicited drinking, an apparent release of ADH and natriuresis The very same effects are obtained by raising the CSF [Na] in this species which has led to the sugge tion that a periventricular sodium sensitive receptor system is involved in the central control of water and salt balance (*cf* Andersson and Olsson 1973) The combined intraventricular infusions of PGE and hypertonic NaCl had much stronger dipsogenic antidiuretic and natriuretic effects than each of these stimuli alone Therefore a possible explanation for the results reported here may be permeability effects of PGE which facilitate the activation of Na sensitive receptors of importance in the central control of fluid balance The recent observation that PGE in

creases the net inward Na^+ movement in the frog skin (Lote Rider and Thomas 1974) appears to lend some support to this idea

This work was supported by the Swedish Medical Research Council (Project 04X 503)

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Acta physiol scand 1975 93 289-294

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Mitochondrial Respiration in the Ventricular Myocardium and in the White and Deep Red Myotomal Muscles of Juvenile Tuna Fish (Thunnus thynnus L)

By

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Received 28 August 1974

Abstract

Modigh M and B Tota *Mitochondrial respiration in the ventricular myocardium and in the white and deep red myotomal muscles of juvenile tuna fish* (*Thunnus thynnus L*) Acta physiol scand 1975 *93* 289-294

The respiratory activity of mitochondria isolated from the outer and inner layers of the ventricular myocard um and from the white and deep red myotomal muscles of juvenile *Th nn s thynnus* has been compared The highest values for the succinate oxidase and succinate cytochrome c reductase activities have been found in the mitochondria of the outer myocard al layer followed by mitochondria of the deep red muscle the inner myocard al layer and the white muscle in that order Differences in mitochondrial NADH-cytochrome c reductase acti ity run parallel in a lower order of magnitude to the differences in the oxidation of succinate This f nding is d scussed in relation to the d fferent metabol c attitudes of the muscle tissues towards anae obic glycolysis The outer myocardial layer of the ju enile tuna ventricle has been shown to ha e a higher metabol c acti ity than the inner layer in contrast to the s tuation in adult ventricular my ocardium

The myotomal muscles in fish are of 2 types red and white The red comprise 5 to 20 per cent of the bulk the proportion varying in relation to the habits of the fish They are generally believed to be used for sustained swimming (George 1962 Marshall 1965 Bone 1966) These muscles oxidize mainly fatty acids and derive their energy from mitochondrial oxidative phosphorylation (Bilinski 1963) They are thus relatively incapable of prolonged anaerobic metabolism as is also cardiac and insect flight muscle (Love 1970) White muscle on the other hand is used for short bursts of speed and may derive energy from anaerobic glycolysis (Black *et al* 1962, Love 1970)

Tuna show an extreme degree of adaptation to pelagic life They have as have also certain other very active fish an additional band of red skeletal musculature the deep red muscle near the spine Furthermore there is in tuna a relatively large proportion of red muscle

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which along with part of the white muscle manifests endothermy (Carey and Teal 1966) Unfortunately information concerning the metabolism in muscle tissue in these most interesting and commercially important fish is fragmentary and incomplete

The present work is concerned with the mitochondrial respiration of white and deep red muscle and with the respiration of the inner and outer layers of the ventricular myocardium in juvenile *Thunnus thynnus* L.

Materials and Methods

Experiments have been carried out on tuna weighing 2 2 5 kg and about 50 cm in length The age of the animals was estimated on the basis of data furnished by Lopez (1965) to be 6 or 7 months Tuna attain sexual maturity at the age of 3 years when they weigh about 15 kg and measure about 1 meter

The fish which die very soon after being taken from the sea were stored at 0 C immediately after the catch The heart and muscle samples were prepared within 4 h after death The myoarchitecture of the cardiac ventricle in fish has two layers The outer cortical layer is a dense and compact structure supplied by oxygenated blood from the coronary arteries the inner spongy layer is mainly perfused by venous blood from the ventricular lumen The heterogeneity of the ventricular myocardium in tuna is very much in evidence and the dissection of the two layers presents no difficulty Specimens of white and deep red muscle from the central third of the trunk of the animals were dissected out from visible connective tissue

All muscle samples were rinsed with cold sea water Mitochondria were prepared from the pool of each myocardial layer obtained from 10 hearts and from the white and deep red skeletal muscle samples by a modification of the method of Bernstein and Wainio (1958) as described by Cariello and Tota (1974) In this way electron transport particle (ETP) preparations containing all the components of the mitochondrial electron transport chain were obtained from each muscle tissue Protein concentrations in the final suspensions of mitochondrial particles were measured with the biuret method (Gornall et al 1948) and adjusted to 15 mg ml with the appropriate amount of 0 1 M phosphate buffer pH 7 4 All biochemical assays were performed on both fresh and stored ETP preparations In the latter case ml aliquots of the fresh ETP preparations were dispensed into ampoules frozen in an acetone dry ice bath and stored at 80 C before use

After two weeks a new ETP preparation was made with a second stock of fish as a control No significant differences in the enzymatic activities as between the two series of ETP preparations were observed

The oxygen consumption reflecting succinate oxidase activity was determined polarographically with a Gilson oxygraph The succinate–cytochrome c reductase and NADH-cytochrome c reductase activities were measured by following the reduction of exogenous ferricytochrome c spectrophotometrically as described by Palcus et al (1969) The concentrations in the reaction mixtures are specified in the legends to the figures Both polarographic and spectrophotometric analyses were performed at 0 5 and 10 C

Results

Fig. 1 shows the oxygen consumption of fresh mitochondria in the presence of succinate Mitochondria from the outer myocardial layer show the higher rate followed by those of deep red skeletal muscle inner myocardial layer and white skeletal muscle in that order Fig. 2 and 3 show succinate–cytochrome c reductase and NADH-cytochrome c reductase activities respectively in the fresh mitochondrial preparations used in polarographic assays The differences in succinate-cytochrome c reductase activity as among the different muscle preparations correspond to those detected in polarographic experiments (Fig 1)

As far as the NADH-cytochrome c reductase is concerned equally high values are found in the outer myocardial layer and deep red muscle followed by the inner myocardial layer and white muscle in that order The differences in the NADH-cytochrome c reductase activity are however of a lower order of magnitude than those recorded for the succinate–

TABLE I Inhibition of succinate oxidase activity of ETP by freezing and thawing

Substrate	Percentage of inhibition (oxygen consumption)		
	Outer layer	Inner layer	Deep red muscle
Succinate = 7 mM (n = 8)	41 ± 3 5	40 ± 2 9	76 ± 3 8
Succinate = 15 mM (n = 7)	38 ± 2 6	40 ± 3 0	72 ± 4 1

For the experimental conditions see Fig 1 The enzymatic activity was evaluated after 4 minutes from the addition of Succinate

cytochrome c reductase and succinate oxidase activities Similar differences in the enzymatic activities were observed in experiments performed at both 20 and 30 C

Since it has been reported that post mortem biochemical changes in the red muscles of fish take place more rapidly than in white muscle (Tomlison and Geiger 1964 Briskey *et al* 1966) the effect of freezing and thawing mitochondria on the succinate oxidase activity of outer and inner myocardial layers and of deep red muscle was studied in order to determine whether the red fibers of these muscle types are affected equally by this treatment and whether the ETP preparations from tuna heart and muscles can be frozen and stored at −80 C without considerable loss of enzymatic activity As may be seen in Table I there is a decrease of about 75 per cent in the enzymatic activity of preparations from deep red muscle while a reduction of about 40 per cent was observed in preparations from the two myocardial layers Unfortunately a lack of appropriate material at the time of these last experiments prevented a statistically valid determination of the degree of these effects on white muscle ETP But it may be stated that the reduction of activity is here less than in deep red muscle

Discussion

The greater oxidative capacity of red muscle as compared with that of white muscle previously reported for several other species of fish is confirmed in tuna Working with fresh

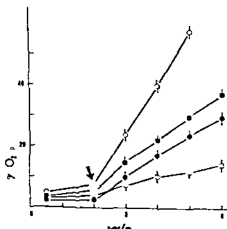


Fig. 1 The oxidation of Succinate by ETP from ○ outer ventricular layer ● inner ventricular layer ■ deep red muscle □ white muscle Reaction mixture contained Phosphate buffer (pH 7 4) 0 1 M ETP 0 80 mg (in terms of protein) sodium succinate 15 mM (added when indicated by the arrow) total volume 1 5 ml T = 25 C Here as well as in Figs 2 and 3 vertical bars are limits of S E. of means. Number of experiments (n) = 9

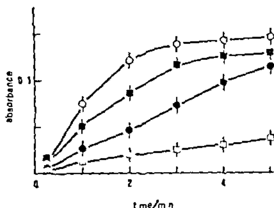


Fig   Succinate Cytochrome c reductase activity of ETP from ○ outer ventricular layer ● inner ventricolar layer ■ deep red muscle □ white muscle The reduction of exogenous Cytochrome c (Horse heart type III Sigma) was followed at 550 nm Reaction mixture contained Phosphate buffer (pH 7 4) 0 1 M ETP 0 80 mg (in terms of protein) KCN 5 10^{-3} mM Cytochrome c 1 mg sodium succinate 10 mM total volume 3 0 ml T = 25 C n = 8

minced preparations of red and white muscle from two species of Pacific tuna (*Katsuwonus pelamis* and *Thunnus obesus*) Gordon (1968) reported that the basal oxygen consumption (oxidation of endogenous substrates) was six times higher in red muscle than in white muscle From a methodological point of view the basal respiration of minced whole muscle may have a different physiological significance differing from that of mitochondrial respiration in the presence of exogenous substrates However the close correlation between our data and those of Gordon is noteworthy We have found that mitochondria from deep red skeletal muscle consume more than three times as much oxygen as do those from white skeletal muscle when the complete electron transport chain is operative (succinic oxidase activity) NADH-cytochrome c reductase activity is twice as great in deep red muscle as in white These lesser differences as compared with those reported by Gordon are probably due to a greater concentration of mitochondria in red muscle (Matsuura and Hashimoto 1954) Nevertheless our data demonstrate a higher respiratory activity in deep red skeletal muscle mitochondria This is in agreement with the electron micrographs of Buttkus (1963) which show a richer system of cristae in red muscle mitochondria and a more tubular structure in white muscle mitochondria in *Ophiodon elongatus*

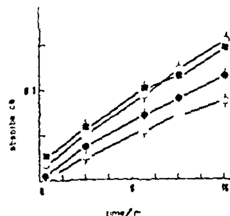


Fig 3 NADH Cytochrome c reductase activity Symbols are those of Fig. Same reaction mixture as in Fig. except for the substrate (succinate replaced by NADH 5mM) T = 25 C n = 8

The NADH–cytochrome c reductase assay affords evidence that the deep red muscle is as active in its capacity for the utilization of NADH as is the outer myocardial layer The differences in NADH–cytochrome c reductase activity as between red and white muscles are less pronounced than the differences in the activities of the succinate branch Since the oxidation of NADH by mitochondria is the only means of coupling the glycolytic pathway of the cytoplasm with the respiratory chain this suggests different metabolic attitudes of the muscle tissues towards anaerobic glycolysis the main source of chemical energy in the white muscle of most teleosts (George and Bokdavala 1964 Love 1970) Even so the oxidative metabolism in tuna white skeletal muscle is not negligible

The high respiratory levels of mitochondria of deep red skeletal muscle are consistent with the very high concentrations of myoglobin found in this muscle (Matsuura and Hashimoto 1959) The high oxidative metabolism in the deep red muscle of tuna may be correlated with the endothermic regulation in this muscle and with the energy required for the great and sustained cruising speeds of these animals It is of interest to mention that in the adult *Thunnus thynnus* Johnston and Tota (1974) have found in the deep red myotomal muscle relatively very high levels of myosin ATPase activity correlated with the intrinsic speed of muscle shortening Braekkan (1956) and Wittemberger (1968) have suggested that the main function of the red muscles is to supply metabolites for the white muscles and they consider the red muscles to be a sort of supplementary liver rather than an organ of locomotion Whatever may be the validity and the applicability of this liver theory the high levels of oxidative metabolism myoglobin concentration and myofibrillar ATPase in these muscles point to a specific role of sustained locomotion for this tissue

It is evident from our data that there are distinct levels of oxidative metabolism in the two myocardial layers of tuna ventricle The outer compact layer served only by the coronary arteries with oxygenated blood (Satchell 1971) and which seems to be the only part of the ventricle with adrenergic innervation (Gannon and Burnstock 1969) and the inner spongy layer which is supplied by venous blood circulating in the lacunae and interstices of the muscle tissue represent two separate muscular compartments Despite this interesting example of muscle heterogeneity in pure myocardial tissue there has hitherto been a complete lack of information about the metabolic and functional characterization of these two layers The very active respiration of the outer layer cannot be explained simply on the basis of unequal oxygen availability as between the two layers since in a previous study (Tota 1970) on adult *Thunnus thynnus* it was demonstrated that homogenates of the inner layer maintain a higher level of oxygen consumption than do homogenates of the outer layer This difference which is the inverse of that found in juvenile tuna has also been shown with the type of mitochondrial preparations employed in the present work (Modigh 1973) The physiological significance of these opposite metabolic patterns in the myocardial layers of juvenile and adult tuna remains to be elucidated

We are indebted to Dr G Carrada of the Zoological Station of Naples for much helpful criticism and discussion

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From the Department of Pharmacology University of Lund Sweden

Acid–Base Changes and Excitation–Contraction Coupling in Rabbit Myocardium I Effects on Isometric Tension Development at Different Contraction Frequencies

By

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Received 7 August 1974

Abstract

Jóhannsson M and E Nilsson *Acid–base changes and excitation–contraction coupling in rabbit myocardium I Effects on isometric tension development at different contraction frequencies* Acta physiol scand. 1975 *93* 295–309

The effects of changes in acid–base parameters on the active force of isolated rabbit papillary muscles were studied at contraction frequencies of 12, 60 and 1 0 min When extracellular pH was lowered from 7 4 to 7 0 and 6 7 in a bathing solution buffered with 10 mM histidine the active force decreased at all contraction frequencies studied After parallel increases of HCO_3^- concentration (up to 47 mM) and P_{CO_2} at a constant extracellular pH of 7 4 the active force of the muscle increased at low and decreased at high contraction frequencies None of these effects can be attributed to catecholamine release or to altered extracellular concentration of ionized calcium The inotropic effects produced by bicarbonate were not reproducible by methyl sulfate (47 mM) or propionate (47 mM) It is concluded that 1 a lowering of the extracellular pH has a negative inotropic effect at all frequencies 2 HCO_3^- has a positive inotropic effect that is most pronounced at low contraction frequencies and 3 CO_2 has a negative inotropic effect exceeding that produced by the mere reduction in extracellular pH The cellular mechanisms involved in the various inotropic effects are discussed

Key words Rabbit heart, isometric force, contraction frequency acidosis, HCO_3^- P_{CO_2}

From previous studies on isolated mammalian myocardial preparations it is not possible to form a clear opinion of how myocardial contractility is affected by different acid–base changes. It has been concluded that extracellular acidosis within the patho-physiological range does not affect contractility (Cingolani *et al* 1970) Extracellular acidosis has also been found to cause a negative inotropic effect (Vaughan Williams and Whyte 1967) Evidence has been presented that a parallel rise in P_{CO_2} and HCO_3^- concentration (at constant extracellular pH) does not alter the mechanical performance of isolated myocardial preparations (Vaughan Williams and Whyte 1967) that it induces a transient negative

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The effects of changes in acid–base parameters on the active force of isolated rabbit papillary muscles were studied at contraction frequencies of 12, 60 and 120/min. When extracellular pH was lowered from 7.4 to 7.0 and 6.7 in a bathing solution buffered with 10 mM histidine, the active force decreased at all contraction frequencies studied. After parallel increases of HCO^- concentration (up to 47 mM) and P_{CO} at a constant extracellular pH of 7.4 the active force of the muscle increased at low and decreased at high contraction frequencies. None of these effects can be attributed to catecholamine release or to altered extracellular concentration of ionized calcium. The inotropic effects produced by bicarbonate were not reproducible by methyl sulfate (47 mM) or propionate (47 mM). It is concluded that 1) a lowering of the extracellular pH has a negative inotropic effect at all frequencies, 2) HCO_3^- has a positive inotropic effect that is most pronounced at low contraction frequencies and 3) CO_2 has a negative inotropic effect exceeding that produced by the mere reduction in extracellular pH. The cellular mechanisms involved in the various inotropic effects are discussed.

Key words: Rabbit heart, isometric force, contraction frequency, acidosis, HCO_3^-, P_{CO}

From previous studies on isolated mammalian myocardial preparations it is not possible to form a clear opinion of how myocardial contractility is affected by different acid–base changes. It has been concluded that extracellular acidosis, within the patho-physiological range, does not affect contractility (Cingolani *et al.* 1970). Extracellular acidosis has also been found to cause a negative inotropic effect (Vaughan Williams and Whyte 1967). Evidence has been presented that a parallel rise in P_{CO_2} and HCO_3 concentration (at constant extracellular pH) does not alter the mechanical performance of isolated myocardial preparations (Vaughan Williams and Whyte 1967), that it induces a transient negative

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inotropic effect (Panmer and Leusen 1968) or causes a permanent depression of contractility (Cingolani *et al* 1970) These discrepancies in results may be attributed to differences in both the myocardial preparation used (atrial or ventricular muscle and/or different species) and in the experimental conditions An obvious difficulty in using a HCO_3^-/CO_2 buffer system is the fact that extracellular pH HCO_3^- or P_{CO_2} cannot be altered individually as at least one of the other two parameters will have to be changed simultaneously Vaughan Williams and Whyte (1967) devised a statistical analysis to determine whether the inotropic effects observed after changes in P_{CO_2} or HCO_3^- concentration are due to alterations of these parameters *per se* or to concomitant changes of extracellular pH A more direct way of obtaining information about inotropic effects induced by extracellular pH-changes would be to use a non bicarbonate buffer solution

In the present work the effects of extracellular acidosis on the mechanical performance of isolated papillary muscles were studied by varying the pH of a solution containing histidine as a buffer With the histidine solution (of pH 7 4) as a control it was also possible to analyse the inotropic effects of parallel changes in HCO_3^- concentration and P_{CO_2} at a constant extracellular pH and to elucidate the effects caused by a separate change of HCO_3^- concentration and P_{CO_2}

Methods

Preparation and mounting

Isolated papillary muscles of rabbits were used The rabbits (weight 0 8–1 5 kg) were heparinized before sacrifice and the heart was immediately removed and opened in oxygenated perfusion solution The papillary muscle and its tendon was dissected from the right ventricle together with a small portion of the ventricle wall near the base of the muscle Platinum loops were firmly tied with silk thread to the tendon and to the piece of the ventricular wall, as closely as possible to the insertions of the muscle The length of the preparations as determined with a microscope (10 magnification) varied within the range 3 to 6 mm and the largest diameter of the muscles was 0 3–0 9 mm

The preparation was mounted horizontally in a jacketed temperature controlled bath (volume 1 5 ml) with the ventricle end connected to a hook and the tendon end attached to a semiconductor strain gauge transducer (Kyowa) The force transducer had a compliance of 1 3 μm/mN and a resonant frequency of approximately 300 Hz with the preparation mounted A linear response was obtained for forces between 0 2 and 50 mN The signal from the transducer was displayed on a Tektronix 502 A oscilloscope and an Elema Schonander Mingograph (frequency response 500 Hz)

The muscle was stimulated by passing current through two pairs of platinum wire electrodes placed on one side of the preparation perpendicular to the long axis of the muscle (distance between two cathodes was 2 mm) The pulse duration was ms and the stimulus intensity was 50 above the threshold value The resting length of the muscle was chosen approximately 5 below the length found to be optimal for active force

Perfusion of muscle bath

Prior to its inflow into the muscle chamber the perfusion solution was equilibrated in a thermostated glass jar with the gas or gas mixture indicated in Table I The pH of the solution was adjusted before the perfusion was started and controlled during the perfusion (see below) The rectangular muscle bath was perfused at a rate of ~4 ml min to give 90 exchange in about 80 s

Temperature control Temperature within the muscle chamber was kept constant at 37 ±0 5 C and was monitored by means of a thermistor in the muscle bath In any given experiment temperature variation was less than ±0 3 C It was specially checked that this value was not exceeded during shift from one buffer solution to another

TABLE I Compos tion of solutions.

mM	zero-HCO_3^-	low HCO_3^-	medium HCO_3^-	high HCO_3^-	high HCO_3^- – low Ca^{++}	$C_2H_5COO^-$	$CH_3SO_4^-$
NaCl	145	142	1‿8	98	98	98	98
$NaHCO_3$		3 4	17	47	47		
$MgCl_2$	1.5	1 5	1 5	1 5	1.5	1.5	1 5
KCl	5	5	5	5	5	5	5
CaCl (added)	2 0	2 0	2.0	2.4	‿0	2 4	‿0
relative (Ca^{++})	1 00	—	1 03	1 0‿	0 75	0 97	0 98
l Histidine	10	10	10	10	10	10	10
d Glucose	10	10	10	10	10	10	10
NaC_2H COO						47	
$NaCH_3SO$							47
Gas	100 O_2	80 O_2 1 CO_2 19 N_2	80 O 5 CO_2 15 N	80 O 13 5 CO 6 5 N	80* O 13.5 CO_2 6 5 N	100 O	100* O
pH	7 4 7 0 6.7	7 4	7 4	7 4	7 4	7 4	7 4

Control solution

Chem als and solutio s The composit on of the different solutions used is given in Table I Chemicals were of analytical grade and the water was double distilled in borosilicate glass. Sodium methyl sulphate was purchased from Hopkin and Williams Ltd The gas mixtures were analyzed by the manufacturer and the concentrations stated were found to be correct to ±0 3 The pH of all solutions was adjusted by ad ding HCl or NaOH During each experiment, pH was controlled intermittently and was not allowed to ary by more than ±0 0 units. Histidine was chosen as a buffer because it was found to reduce the Ca^{++} concentrat on of the solutions to only a small degree In 3 experiments omission of h stidine (10 mM) from the med m HCO_3^- solution i creased the Ca + concentration by 6 5 (range 5 7–7 5) In 4 con trol experiments it was demonstrated that the add tion of 10 mM h st dine to the med um HCO_3^- solut on d d not alter the isometric force of papillary muscles at contraction frequencies 1‿, 60 and 1 0 beats min

D te mination of Ca^{++} conc trat on

It is well known that many ions can bind calcium and therefore the calcium ion concentration of the solu tions was measured with a calc um sensitive electrode (Orion model 99 0) (Moore 1970) In Table I the Ca concentration of the various solutions is gi en in units of the Ca + concentration present in the zero – HCO_3^- solution The Ca^{++} concentration of the 'zero – HCO^- solut on was found not to be influ enced by pH alterat ons with n the range 6 7–7 4 The Ca concentration was nearly ident cal in the zero-HCO^- solut on and n the medium HCO_3^--solution However an increase in HCO_3^- concentration from 17 to 47 mM reduced Ca^{++} concentration by approx ‿5 In order to reach the same Ca^+ concentration in the h gh HCO as in the zero-HCO_3^- solution extra $CaCl_2$ was added to the latter solut on.

R s p i at of a mals

Reserpine (Serpasil[®] CIBA) was g ven intraperitoneally 4 mg/kg, 18–‿4 h before the sacrifice of the an mal. It hs been demonstrated that most of the noradrenaline of sympathet c nerve terminals of the heart d sappear after such a pretreatment of the animals (Spilker and Cervoni 1969)

Experimental procedures and evaluation of data

An equilibration time of 60 min was allowed before the experiment was started During the equilibration period the preparation was paced to contract at a frequency of 60/min This stimulation frequency was also used during the whole experiment except for brief periods of stimulation at frequencies of 12 or 120/min

After the equilibration period the isometric twitch response was recorded at 12 60 and 120 contractions/min in the control solution (pH 7 4) in the test solution and in the control solution again The muscle bath was perfused with a given solution for at least 15 min before changing the stimulation frequency to 12 or 120/min The active force at a frequency of 12/min was measured after attainment of steady state responses which occurred 3–5 min after switching to this frequency At 120 stimulations/min the optimum isometric tension was measured as generally no steady state was reached at this frequency The optimum occurred within 1 3 min after altering the stimulation rate from 60 to 120/min The steady state tension at 60 stimulations/min was recorded before and after determining the isometric twitch amplitude at a frequency of 12 or 120/min

Thus in each solution the preparation was paced in the following order 60 12 60 120 and 60/min The isometric twitch tension obtained at 12 and 120 contractions/min in a given solution is given as a fraction of the mean value of the twitch amplitude at 60 stimulations/min determined before and after the test period

In order to compare the peak isometric twitch tension in the control solution and in any of the test solutions the following procedure was used The mean value of the twitch tension obtained in the control solution at 60 stimulations/min before and after perfusion with the test solution was taken as reference Mean values of peak twitch tension at 12 and 120 stimulations/min in the control solution as well as twitch amplitudes at all frequencies in the test solution are given as fractions of the reference tension In this way slow changes of contractility of the muscles with time could be allowed for Only in experiments with the high HCO_3 solution was a slight but consistent irreversible deterioration of the preparation observed

In the figures isometric tension is shown as mean ± S E The statistical evaluation of inotropic effects of a test solution at a given contraction frequency was based upon the difference between the isometric force in the test solution and that in the control solution A *t* test based on paired observations was used

Results

A Influence of extracellular pH on the isometric twitch

In the analysis of the effects of extracellular pH-changes on isometric twitch tension solutions buffered with histidine alone at pH 7 4 7 0 and 6 7 were used The calcium concentration of the histidine solutions did not vary with pH within the range studied (see Methods) Measurements at pH 7 0 and 6 7 were started 15–30 min after a change from pH 7 4 When extracellular pH was lowered from 7 4 to 7 0 or 6 7 the active force of the papillary muscles (measured at 60 stimulations/min) diminished slowly and reached a steady state 10–15 min after alteration of pH Recordings obtained up to 90 min after a lowering of pH revealed no further change in peak isometric tension Fig 1 shows the peak isometric force of the muscles at stimulation frequencies 12 60 and 120/min The mean value of the isometric tension at a stimulation frequency of 60/min in the solution of pH 7 4 is taken as unity (see Methods) As demonstrated in Fig 1 a lowering of pH within the range 7 4–6 7 caused a reduction of the isometric tension at all contraction frequencies studied However extracellular pH-changes did not alter the relative time course (kinetics) of the isometric twitch Fig. 2 shows traces of typical isometric twitches at pH 7 4 and 6 7 Note that time to peak tension and the duration of the relaxation phase is not significantly affected by the pH change

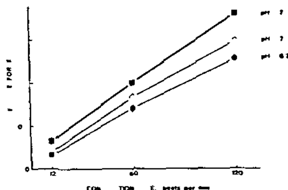


Fig. 1 The peak isometric force of rabbit papillary muscles at different extracellular pH as a function of contraction rate The force at pH 7 4 and 60 beats min is taken as unity Symbols denote mean±S E. for 7 expts. The active force is significantly diminished when pH is lowered from 7 4 to 7.0 or to 6 7 (* $p<0.05$ ** $p<0.01$ *** $p<0.001$)

B *Effects on the isometric twitch of parallel changes in HCO_3 concentration and P_{CO_2} at constant extracellular pH*

At a constant contraction frequency of 60/min, an increase in both (HCO_3^-) and P_{CO_2} at pH 7 4 induced specific changes in contractile force as illustrated in Fig. 3 There was an initial rapid reduction of peak isometric force which passed within two min and was succeeded by a positive inotropic effect The latter effect persisted in low and medium HCO_3^- solutions After approximately 3 min in the high-HCO_3^- solution on the other hand peak isometric force decreased again and reached an approximate steady state after 20 min

In Fig. 4 are shown the effects of parallel changes in HCO_3^- concentration and P_{CO_2} at pH 7 4 on peak twitch tension at contraction frequencies 12, 60 and 120/min All results refer to measurements obtained after 15–30 min in the respective HCO_3^- solution. As can be seen in the low HCO_3^- solution there was a slight increase in active force at all stimulation frequencies studied The effect was statistically significant only at a contraction frequency of 60/min In medium HCO_3^- and high HCO_3^- solutions peak twitch tension was enhanced at 12 contractions/min and decreased at 120 contractions/min as compared to the situation in the control solution Indeed in the high HCO_3^- solution no increase in active force with increasing contraction frequency occurred.

The effects of parallel changes in P_{CO_2} and HCO_3^- concentration on peak twitch tension involve alterations in both the rate of force development and the time to peak twitch tension This is demonstrated in Fig 5 A and B which show isometric twitch responses at contraction frequencies 60 and 12/min at various times after changing the perfusion from the control solution of pH 7 4 to the high HCO_3^- solution of the same pH At both contraction frequencies time from stimulus to peak tension (TPT) shortened successively in



Fig. Redrawn oscilloscope traces of isometric force at pH 7 4 and 6 7 Contraction rate 60 beats/min. Note that the time to peak tension and the duration of contraction are unaffected by the pH change

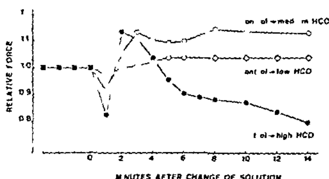


Fig. 3 Isometric force of rabbit papillary muscles during the first 14 min after a change in perfusing solution At zero time the control solution is replaced by one of the bicarbonate solutions In each case the points denote the mean of 5 expts at a contraction rate of 60 beats min. Note the initial reduction of isometric force and the secondary increase in force which persists in low HCO_3^- and medium HCO^- solutions.

the high HCO_3 solution The maximum rate of force development (dP/dt_{max}) increased steadily to a maximum at 12 contractions/min whereas at a frequency of 60 beats/min dP/dt_{max} was enhanced during the initial 2 to 4 min after switching to the high HCO_3 solution after which it decreased

C Inotropic effects produced by changes in either HCO_3^- concentration or P_{CO}

When only HCO_3 concentration or P_{CO} of the perfusion solution is altered extracellular pH is also varied If however the effects of extracellular pH changes *per se* are known or another solution of the same pH can be used as a control the specific influence of HCO_3)

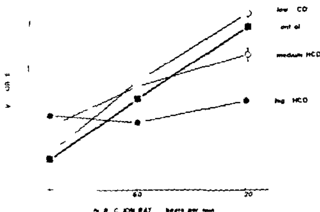


Fig. 4 Peak isometric force of rabbit papillary muscles as a function of contraction rate in the control solution (zero-HCO_3 pH 7 4) and n solutions with different concentrations of HCO_3 and different P_{CO_2} all solutions having a pH of 7 4 The force in the control solution at 60 beats min is taken as un t Symbol denote mean ± S E for 5 8 expts Note that acti e force in the medium HCO_3 and h gh HCO solutions is significantl ncreased at 1 beats min and significantly decreased at 120 beats m n as compared to recordings in the control solut on

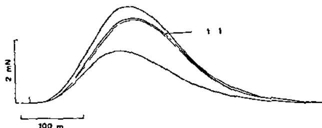


Fig 5 A Redrawn oscilloscope traces of isometric force at 60 beats/min taken at different times after re placing the control solution with the high HCO_3^- solution The trace just below the control is taken 1 min the uppermost trace 3 min and the lowest trace 15 min after change of solution Note the increased rate of force development and shortened time to peak tension after 3 min and also the shortened time to peak tension after 15 min

and P_{CO} alterations may be deduced The following 2 types of experiments were undertaken in order to investigate this problem

1 Peak isometric force was determined as previously described at contraction frequencies 12 60 and 120/min in zero HCO_3 solution (containing 10 mM histidine) *of pH* 7 8 The preparation was then immersed in the medium HCO_3^- solution and paced to contract at 60/min When perfusion with this solution was started its aeration was changed from the gas mixture containing 5 % CO_2 and 80 % O to pure O Hence pH of the solution slowly rose and after approximately 10 min a pH of 7 8–7 9 was reached Isometric con tractions of the papillary muscle were recorded at 12 60 and 120 contractions/min 20–25 min after changing to the medium HCO_3 solution with 100 % O_2 In Fig 6 are illustrated mechanical recordings in the zero HCO_3 solution of pH 7 8 and in the medium HCO_3 solution of pH 7 8 As can be seen a positive inotropic response occurred in the medium HCO_3 solution which was more pronounced at low than at high contraction frequencies The effects described were fully reversible within a time period of 1 hour

2 In the other series of experiments the isometric twitch of papillary muscles was meas ured in medium HCO_3 solution (aerated with 5 % CO pH 7 4) at contraction frequencies 12 60 and 120/min The aeration of the solution was then switched from 5 to 13 5 % CO_2 and consequently the pH of the medium HCO_3^- solution was lowered After approximately 10 min it reached steady state at 7 0 When the muscle bath had been perfused with this solution for another 10 min isometric twitches were again recorded at 12 60 and 120



Fig. 5 B The same as in Fig 5 A except that the contraction rate is 12 beats min The middle trace is taken 5 min and the uppermost trace 15 min after changing solutions Note the increase in force and rate of force development, and the shortening of time to peak tension as compared to the trace in the control solution

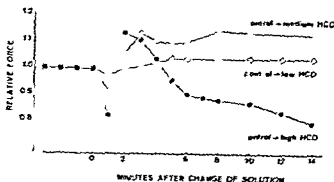


Fig. 3. Isometric force of rabbit papillary muscles during the first 14 min after a change in perfusing solution. At zero time the control solution is replaced by one of the bicarbonate solutions. In each case the points denote the mean of 5 expts. at a contraction rate of 60 beats/min. Note the initial reduction of isometric force and the secondary increase in force which persists in "low HCO_3^-" and "medium-HCO_3^-" solutions.

the high-HCO_3 solution. The maximum rate of force development (dP/dt_{max}) increased steadily to a maximum at 12 contractions/min, whereas at a frequency of 60 beats/min dP/dt_{max} was enhanced during the initial 2 to 4 min after switching to the high HCO_3 solution after which it decreased.

C. *Inotropic effects produced by changes in either HCO_3 concentration or P_{CO_2}*

When only HCO_3 concentration or P_{CO_2} of the perfusion solution is altered, extracellular pH is also varied. If however the effects of extracellular pH changes *per se* are known or another solution of the same pH can be used as a control the specific influence of HCO

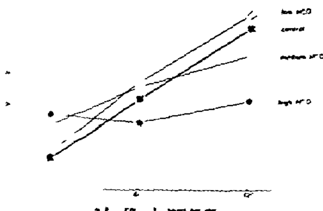


Fig. 4. Peak isometric force o[illegible] papillary muscles as a function of contraction rate in the control solution (ZETO-HCO [illegible] pH [illegible]) a[illegible] solutions with different concentrations of HCO and [illegible]. [illegible] all solutions ha[illegible] a pH of 7.4. The force in the control solution at 60 beats/min is taken as [illegible] denote mean ± S.E. for 5–8 ex[illegible] that active force in the medium-HCO and high HCO [illegible] tions is sig[illegible] increased at [illegible] beats/min and significantly decreased at 1[illegible]0 beats/min as com[illegible] to recordings in the control solution.

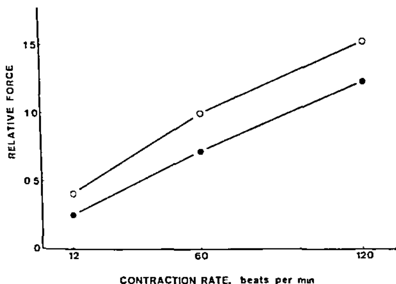


Fig. 7 Peak isometric force as a function of contraction rate at different Pco and constant HCO_3^- concentration. The diagram shows the isometric force in the medium-HCO_3^- solution bubbled with 5 CO_2 (pH 7.4) (○) and bubbled with 13 5 CO (pH 7 0) (●) When P_{CO_2} is increased the isometric force is diminished at all contraction rates studied Symbols denote the mean of 3 expts

30 min exposure to this solution As can be seen perfusion with the high HCO_3 –low Ca + solution altered the frequency–force relation in very much the same manner as previously described for the high-HCO_3^- solution, *i.e* a significant increase in peak twitch tension occurred at a contraction frequency of 12/min and a reduction of force relative to the

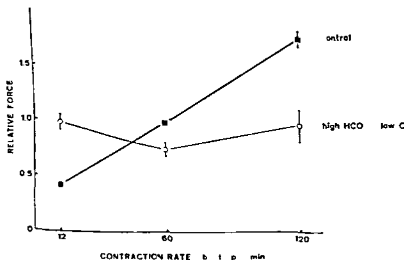


Fig. 8 Peak isometric force as a funct on of contraction rate in control solut on and in h gh HCO^-–low Ca solut on. The isometric force in the h gh HCO_3^-–low Ca^{++} solution is significantly greater at 1. beats/m n and s gn ficantly lower at 1 0 beats m n as compared to the force in the control sol tion Symbols denote mean ± S E. for 5 expts.

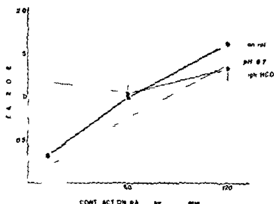


Fig 17 Peak isometric force of papillary muscles from reserpinized rabbits as a function of contraction rate When pH is lowered from 7 4 to 6 7 the force is diminished at all contraction rates In the high HCO_3 solution the force is increased at 17 beats/min and decreased at 120 beats/min as compared to the control solution Symbols denote mean ± S E for 5 expts

In order to describe the mechanisms of inotropic effects induced by acid–base alterations knowledge of intracellular pH regulation in myocardial cells would be required Information on this point is however still very incomplete It is generally held that pH of the cytoplasm in muscle cells is approximately 7 0 (Waddell and Bates 1969) Both *in vivo* and *in vitro* studies indicate that in skeletal muscle increasing P_{CO_2} rapidly lowers intracellular pH whereas addition of highly ionized acids or bases (such as HCl or Na HCO_3) to the extracellular medium does not cause a significant change of intracellular pH within an hour after alteration of extracellular pH (Brown and Goott 1963 Williams *et al* 1971) *In vivo* studies on myocardium (Benson *et al* 1965 Clancy and Brown 1966) have shown that a rise of P_{CO_2} lowers intracellular pH and that the apparent buffer capacity (measured as $d(HCO_3^-)/dpH$ intracellularly) is greater in myocardium than in skeletal muscle Little direct information seems to be available however as to how changes in extracellular HCO_3 concentration and extracellular pH affect intracellular pH in cardiac muscle within time periods comparable to those used in the present study

Extracellular acidosis

In the present work the influence of extracellular acidosis on contractility of isolated papillary muscles could be studied without interference from extracellular changes in HCO_3 concentration or P_{CO_2} It was demonstrated that a lowering of extracellular pH within the range 7 4–6 7 reduced peak isometric twitch amplitude without altering the kinetics of the twitch both in normal and in catecholamine depleted muscles This finding is at variance with results obtained by Cingolani *et al* (1970) on cat papillary muscles but in accordance with conclusions reached by Vaughan Williams and Whyte (1967) Mead and Little (1967) and Pannier and Leusen (1968) on atrial strips and papillary muscles of rabbits The conclusions reached in the previous studies were however based on indirect information from studies in solutions buffered with HCO_3 and CO_2

A reduction of extracellular pH from 7 4 to 6 7 is not associated with any change of the duration or rate of rise of the cardiac action potential but causes a slight significant decrease in its conduction velocity (Jóhannsson and Nilsson 1975) As has been pointed out above intracellular pH is probably unaffected by extracellular acidosis and therefore does

not provide an explanation for the negative inotropic effect Despite the insignificant changes in the action potential it seems possible that the negative inotropic effect of extracellular acidosis can be ascribed to a small reduction of the Ca flux into the cell during the action potential (see Discussion in Johannsson and Nilsson 1975)

Changes in P_{CO} and HCO_3 concentration

In the present study increased HCO_3 concentration caused a positive inotropic effect more pronounc d at low than at high contraction frequencies whereas increased P_{CO} lowered myocardial contractility at all frequencies

The transient changes of the isometric twitch response observed after a parallel increase in HCO_3^- concentration and P_{CO} probably reflect different time courses of the individual effects produced by HCO_3^- and CO_2 the initial reduction in peak amplitude of the twitch being caused by increased P_{CO} the subsequent increase in rate of rise of tension and shortening of the time to peak twitch tension being attributable to increased HCO_3^- concentration The permeability of CO is thought to be greater than that of HCO_3 (see Kammermeier and Rudroff 1972) and therefore it is to be expected that the negative inotropic effect caused by increased P_{CO} occurs very early after changing to a medium with higher P_{CO} (*cf* Fig 3) Transient depression of myocardial contractility following a P_{CO} increase has been observed on cat papillary muscle (Pannier and Leusen 1968) frog ventricle (Lorcovic 1966) and isolated guinea pig hearts (McElroy *et al* 1958 Kammermeier and Rudroff 1972)

An increase of P_{CO} within the range analysed in the present study does not affect the duration rate of rise or propagation velocity of the action potential (Jóhannsson and Nilsson 1975) However a lowering of intracellular pH brought about by *e g* increased P_{CO} may influence several processes beyond the excitation of the cell membrane The apparent Ca + affinity of the sarcoplasmic reticulum of cardiac muscle increases with decreasing pH within the range 7 5–6 0 (Nakamaru and Schwartz 1970 Entman *et al* 1972) It has therefore been suggested (Nakamaru and Schwartz 1970) that lowering the intracellular pH within this range can lead to a reduction of the amount of Ca^+ released after excitation Still another mechanism whereby intracellular acidosis may lower myocardial contractility is provided by the finding that a decrease in pH within the range 7 0–6 0 decreases the ATPase activity and tension development of the actomyosin system in glycerol extracted myocardial preparations (Schadler 1967)

The frequency-dependence of the inotropic effects of HCO_3 does not seem to have been reported previously The positive inotropic effect of HCO_3 is attributable to this ion itself and not to an increased buffer capacity of the external medium This conclusion is supported by the finding that an increase in buffer capacity of the perfusion solution produced by addition of 10 mM histidine does not affect the contractile response (see Methods) Furthermore if an increased extracellular buffer capacity caused a positive inotropic effect by facilitating the transport of acids from intra to extracellular space this effect would be less pronounced at low than at high contraction frequencies as intracellular acidosis is more likely to occur in the latter situation

The shortening of the time to peak twitch tension seen after a parallel rise of HCO_3 concentration and P_{CO}, indicates a reduction in the time during which the flow of Ca^{++}

into the myofibrillar space exceeds the rate at which activator calcium is eliminated The alteration in the kinetics of the twitch is paralleled by a shortening of the duration of the action potential brought about by the raised HCO_3 concentration (Jóhannsson and Nilsson 1975) This accords with the idea that during steady state conditions the time course of the action potential controls the duration of the release of activator Ca (Kavaler 1959 Edman *et al* 1966 Morad and Trautwein 1968 Antoni *et al* 1969) The secondary negative inotropic effect at high contraction frequencies in high HCO_3 solution seem to indicate that the abbreviation of the action potential in this solution also reduces the net release of activator calcium

Membranes of muscle cells are generally considered as less permeable to HCO_3 than to Cl although specific information on this point is scarce (see Mainwood and Lucier 1972) The present experiments with propionate and methylsulfate are of great interest as they suggest that a mere replacement of Cl with a less permeable anion cannot mimic the inotropic effects of the HCO_3 ion

The authors wish to thank Professor K A P Edman for helpful discussions and for criticism of the manuscript and Miss Christina Olsson and Mrs Karin Johansson for excellent technical assistance The work was supported by grants from the Swedish Medical Research Council (project No 04X 184) and from the Medical Faculty University of Lund

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Acta physiol scand 1975 93 310-317
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Acid-Base Changes and Excitation-Contraction Coupling in Rabbit Myocardium II Effects on Resting Membrane Potential, Action Potential Characteristics and Propagation Velocity

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Received 7 August 1974

Abstract

Jóhannsson M and E Nilsson *Acid-base changes and excitation-contraction coupling in rabbit myocardium II Effects on resting membrane potential action potential characteristics and propagation velocity* Acta physiol scand 1975 *93* 310-317

The effects of changes in acid-base parameters on the resting membrane potential action potential characteristics and propagation velocity were studied in isolated rabbit papillary muscles Lowering extracellular pH from 7 4 to 6 7 in a bathing solution buffered with 10 mM histidine did not alter the resting membrane potential or action potential characteristics but caused a slight reduction in propagation velocity A parallel increase in HCO_3^- concentration (up to 47 mM) and P_{CO_2} at a constant extracellular pH of 7 4 caused a substantial decrease in action potential duration but did not alter the resting membrane potential or propagation velocity The decrease in action potential duration was caused by the increase in HCO_3 concentration Propionate (47 mM) caused a shortening of the action potential which was of the same magnitude as for HCO_3 but methylsulfate (47 mM) did not have this effect The possible influence of these changes on the inotropic state of the myocardium and the cellular mechanisms involved are discussed

Key words Rabbit heart membrane potential action potential propagation velocity acidosis HCO_3 P_{CO_2}

The effects of acid-base changes on electrophysiological parameters in myocardium have previously been studied in rabbit atria (Vaughan Williams and Whyte 1967) and frog ventricle (Lorković 1966) There is still lack of information however as to the effects of acid base changes on electrophysiological parameters of mammalian *ventricular* myocardium The present study performed on rabbit papillary muscle was undertaken in order to fill this gap The effects of acid-base changes on resting membrane potential action potential characteristics and propagation velocity were studied using the same solutions and a similar experimental approach as that described in the preceding paper (Jóhannsson and Nilsson 1975) A preliminary account of some of the results has been given previously (Jóhannsson and Nilsson 1971)

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Methods

The experiments were performed on isolated papillary muscles of rabbit hearts A deta led description of the d ssection and mounting of the preparation has been given in the preceding paper (Johannsson and Nilsson 1975) The electrical recordings described in the present paper were generally performed on the same muscles as used for the mechanical measurements in the previous article

Record ng techn ques

I t acellular reco di gs of transmembrane potentials were obtained by conventional electrophysiological techniques Glass microelectrodes filled with 2.5 M KCl, were used The resistance of an electrode was between 10 and 25 MΩ, and the tip potential was less than 5 mV The signals from the intracellular electrode and a reference electrode in the grounded muscle chamber were fed differentially into an amplifier with high input impedance and capacitance neutralisation (Holmer and Lindstrom 1972) The amplifier output was displayed on a Tektronix 507A oscilloscope, and photographed on 35 mm film For measurements of *maximum depola i ation veloc ty* (dV/dt_{max}) the ampl f er output was differentiated by means of an operational amplifier circuit (Philbrick, SQ-10a) The differentiator was found to have a linear response over the range 5–1 000 V/s In each solution, the following parameters were measured in 4–6 different cells resting membrane potential action potential duration at −20 mV and −60 mV level, and dV/dt_{max} *Propagation veloc ty of the act on potential* was measured by stimulating the muscle at one end with a punctate electrode and measuring the time between the stimulus artefact and dV/dt_{max}, when the microelectrode was inserted at various distances from the stimulating electrode In each solution 8–12 measurements were made for d fferent distances between the stimulating electrode and the intracellular m croelectrode The distance was first increased and then decreased in 0 5 mm steps and was measured to the nearest 0 05 mm by means of a Zeiss stereo m croscope (70 × magnif cation) provided with an ocular micrometer The slope of the corresponding regression l ne gave the propagation velocity (see Fig. 2)

S l tions

The compo ition of the different solutions is given in detail in Table I of the preceding paper (Johannsson and Nilsson 1975)

Statist cal evaluation

As in the preceding paper statist cal e aluation of the data is based on the t test for paired observat ons

Results

Unless otherwise stated the data presented here were obtained at a stimulation frequency of 60/min As can be seen in Table I of the preceding paper (Johannsson and Nilsson 1975) the concentration of ionized calcium was the same in all the different solutions On the other hand the concentration of Cl^- varied when the concentration of HCO was changed In 4 expts histidine was omitted from the medium HCO_3 solution which normally contained 10 mM histidine This change of the bathing solution caused no detectable difference in the resting membrane potential action potential duration or dV/dt_{max}

A *Influence of extracellular pH*

Extracellular pH was lowered by adding HCl to the zero-HCO_3^- solution which was buffered with 10 mM histidine only The effects of lowering pH from 7 4 to 6 7 are shown in Table I As can be seen resting membrane potential action potential duration and dV/dt_{max} were unaffected by extracellular pH within the range studied whereas propagation velocity was slightly but significantly reduced by extracellular acidosis Traces of typical action

TABLE I Effects of alterations in extracellular pH on electrophysiological parameters of rabbit papillary muscles. The solutions were buffered with 10 mM histidine Mean ± S E. n is the number of experiments. P degree of probability N S., not statistically significant

	pH 7 4	pH 6 7	Mean difference	P	n
AP duration at − 20 mV	14[illegible]0 ± 10 2	137 2 ± 12 9	− 4 8 ms	N S	8
AP duration at − 60 mV	189 7 ± 9 7	187 0 ± 13 3	− 2 7 ms	N S	8
Overshoot	21 8 ± 1 0	20 3 ± 1 6	− 1 5 mV	N S	8
Resting potential	− 75 8 ± 0 9	− 75 7 ± 0 7	− 0 1 mV	N S	8
dV/dt_{max}	130 6 ± [illegible]4 5	130 9 ± 25 5	+ 0 3 V/s	N S	6
Propagation velocity	0.51 ± 0 04	0 46 ± 0 04	− 0 05 m/s	<0 0[illegible]	5

potentials at pH 7 4 and 6 7 are shown in Fig. 1 Results of one typical experiment illustrating the determination of the conduction velocity at pH 7 4 and 6 7 are illustrated in Fig 2. All measurements of Table I were obtained 15–60 min after changing the pH In some experiments impalements were also made during the first 15 min after changing the perfusion solution to see if any transient changes occurred, but no such changes could be detected.

B *Effects of simultaneous changes in HCO_3 concentration and P_{CO_2} at constant extracellular pH*

All solutions used in these experiments had the same pH (7 4) but varied with respect to HCO_3 concentration and P_{CO_2} In each experiment the solution buffered with 10 mM histidine alone (zero-HCO solution) was used as a control

The low HCO_3 solution (3 4 mM HCO_3 1 % CO_2) did not have any significant effect on the recorded electrophysiological parameters The effects of changing from control solution to medium HCO_3 (17 mM HCO_3 5% CO_2) and high HCO_3 (47 mM HCO_3 13 5 % CO) solutions are shown in Table II and Table III respectively A significant decrease in action potential duration occurred in both solutions This shortening is about 20 % in the medium-HCO solution and 40–50 % in the high HCO solution In about 1/3 of the experiments in high HCO_3 solution there was a complete disappearance of the plateau

TABLE II Electrophysiological parameters of rabbit papillary muscles recorded in control solution (zero-HCO) and in medium-HCO_3 solution (17 mM HCO 5 % CO_2) Mean ± S E n is the number of experiments P degree of probability N S not statistically significant

	Control	Medium-HCO	Mean difference	P	n
AP duration at − 0 mV	137 5 ± 10 1	105 7 ± 10 0	− 31 8 ms	p 0 0	8
AP duration at − 60 mV	18 1 ± 1[illegible]	145 0 ± 11 5	− 37 1 ms	p 0 0	8
Overshoot	1 7 ± 1 1	[illegible]3 0 ± 1 1	1 mV	N S	8
Resting potential	75 5 ± 1 3	4 8 ± 0 7	0 6 mV	N S	8
dV/dt_{max}	153.8 ± 16 1	158 4 ± [illegible] 4	4 5 V/s	N S	5
Propagation velocity	0 43 ± 0 05	0 48 ± 0 03	0 m/s	N S	5

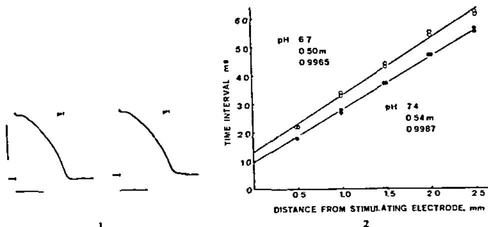


Fig 1 Traces from a typ cal experiment showing action potential recordings in the zero-HCO_3^- solution at pH 7 4 and 6 7 Horizontal line 100 ms. Vertical line 50 mV upper end denotes zero potential

Fig. 2 A typical experiment showing the determination of propagat on velocity in a rabbit papillary muscle The abscissa shows the distance between a punctate stimulating electrode and a recording intracellular m croelectrode The ordinate shows the time between the stimulus artefact and dV/dt_m r is the correla tion coefficient for the regression line and V is the propagat on velocity Recordings were made at pH 7 4 (●) and pH 6 7 (○) Each symbol represents a single measurement

phase of the action potential (Fig 3) No changes in resting membrane potential or in the rate of rise and amplitude of the action potential were observed

The values given in Tables II and III were obtained at the standard stimulation frequency of 60/min In 4 expts the above effects were also studied at stimulation frequencies of 12 and 120/min Also under these conditions the action potential duration was found to be approximately 20 and 45% shorter in the medium and high HCO_3 solutions respec tively than in the control solution

The results presented in Tables II and III all refer to steady state responses in the respec tive solutions Attempts were also made however to study the development of the action potential change after an alteration of the bathing medium. Fig 3 shows the shape of the action potential in the control solution and at different times after shifting to the high

Table III Electrophysiolog cal parameters of rabb t pap llary muscles recorded in control solution (zero-HCO_3^-) and in high HCO_3^- solut on (47 mM HCO_3^- 13 5 CO_2) Mean ± S E n is the num ber of experiments P degree of probab l ty N S not statistically sign f cant

	Cont ol	High HCO_3^-	Mean differences	P	n
AP durat on at − 0 mV	144 5 ± 5 7	76 1 ± 1 2	−68 3 ms	p 0 01	7
AP duration at −60 mV	19 1 ± 5 0	11_1 ± 13 9	−80 0 ms	p < 0 01	7
Overshoot	_5 9 ± 4 9	7 4 ± 4 7	+ 1 5 mV	N.S	7
Resting potential	−75 1 ± 0 6	−75 7 ± 0 2	+ 0 6 mV	N S	7
dV/dt_{max}	9 6 ± 10 1	94_ ± 8 7	+ 1 6 V s	N S	5
Propagation velocity	0 45 ± 0 03	0 46 ± 0 03	− 0 01 m s	N S	5

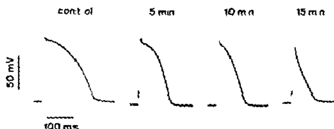


Fig. 3 A typical experiment showing the progressive shortening of the action potential after a change from the control (zero-HCO_3^-) solution to the high HCO_3^- solution The action potential is shown 5 10 and 15 min after a change to the latter solution The complete absence of a plateau seen after 15 min in this experiment was observed in about 1/3 of the experiments Zero potential is denoted by the upper end of the 50 mV calibration line

HCO_3 solution It can be seen that the decrease in action potential duration developed gradually The duration reached a steady state after 15–20 min

C *Effects produced by changes in either HCO_3^- concentration or P_{CO}*

It was of interest to investigate in a more direct way the specific influence of changes in HCO_3^- concentration and P_{CO} on the action potential duration The following 2 types of experiments were undertaken using the procedure described in detail in the preceding paper (Johannsson and Nilsson 1975 page x)

1 Action potentials were measured in the zero-HCO_3 solution (buffered with 10 mM histidine) of *pH 7 8* and in the medium HCO_3 solution aerated with pure O_2 (*pH 7 8–7 9*) These two solutions thus differed only as to their HCO_3 and Cl^- concentrations In 3 experiments of this kind the action potential duration was found to be 10–18% shorter in solution containing 17 mM HCO_3 than in the zero-HCO solution This is comparable h the 20% shortening of the action potential that was found after changing from the ontrol solution to a medium HCO_3 solution aerated with 5% CO_2 (Table II)

2 In the other series of experiments action potentials were recorded in the medium HCO solution aerated with 5% CO_2 (pH 7 4) or with 13 5 % CO_2 (pH 7 0) *Hence these* solutions had the same HCO_3 concentration but differed with respect to pH and P_{CO_2} As the resting membrane potential and the action potential duration are unaffected by alterations in pH within this range (Table I) any change in action potential configuration that might have occurred would have been due to an alteration of P_{CO} alone In four experiments this variation of P_{CO_2} was found not to affect the duration of the action potential

The results described above thus indicate that the reduction in action potential duration observed when P_{CO} and HCO_3 concentration of the perfusing solution are increased at constant extracellular pH can be entirely attributed to the altered HCO_3 concentration

D *Effects of changes in anion composition of extracellular fluid*

The possibility existed that the shortening of the action potential duration recorded in medium HCO and high HCO solutions (section B) was in fact caused by a decrease in Cl concentration In order to test this point measurements of the resting membrane potential and the action potential were made in solutions where 47 mM Cl had been

replaced by an equimolar concentration of $CH_3SO_4^-$ or $C_2H_5COO^-$ (see further Table I in Johannsson and Nilsson 1975) Measurements were also carried out on the same preparations in the control solution (zero-HCO_3^-)

In 4 expts no difference in action potential duration was observed between recordings made in the $CH_3SO_4^-$ solution and in the control solution On the other hand as demonstrated by 4 other expts the action potential duration was 40–50 % shorter in the $C_2H_5COO^-$ solution than in the control solution This shortening of the action potential is of the same magnitude as observed in the high HCO_3^- solution (containing 47 mM HCO_3^-) (see Table III) No change of resting membrane potential occurred in the $CH_3SO_4^-$ solution or in the $C_2H_5COO^-$ solution

Discussion

Extracellular acidosis

When pH was lowered from 7 4 to 6 7 the only change observed was a decreased conduction velocity This finding agrees with results obtained by Vaughan Williams and Whyte (1967) in studies of isolated rabbit atria These authors however also observed a decline in resting potential and a diminution of dV/dt_{max} findings which were not observed in the present investigation The unchanged duration of the action potential when pH was lowered is in agreement with the results of Lorkovic (1966) in frog ventricular strips but Vaughan Williams and Whyte (1967) found a small prolongation of the tail of the action potential in rabbit atria There is no obvious explanation for these differences in results

As discussed in the preceding paper (Johannsson and Nilsson 1975) the negative inotropic effect of an increased extracellular H^+ concentration is probably due to altered properties of the cell membrane It has been shown that pH affects the ionic conductance in nerve fibres (Hille 1968 Hafemann 1969) and the possibility exists that low extracellular pH causes a decrease in calcium conductance and thus a reduced inward calcium current in myocardial cells It has in fact been demonstrated that a decrease of extracellular calcium concentration or the addition of substances that diminish calcium conductance do hardly affect the shape of the action potential in mammalian myocardial cells (Tritthart *et al* 1973 a) It thus seems conceivable that the negative inotropic effect of extracellular acidosis in spite of the unaffected shape of the action potential is due to a decrease in calcium conductance

Changes in HCO_3^- concentration and P_{CO_2}

In the present study a substantial shortening of the action potential duration attributable to the bicarbonate ion was observed after a parallel increase of P_{CO_2} and extracellular bicarbonate ion concentration In frog ventricle substitution of $NaHCO_3$ for 110 mM NaCl in the extracellular fluid has been shown to cause a substantial shortening of the action potential (Lorkovic 1966) An increase of extracellular bicarbonate ion concentration from 5 to 24 mM at constant extracellular pH was found not to affect the action potential duration in rabbit atrium (Vaughan Williams and Whyte 1967) The influence of the bicarbonate ion on the action potential of the mammalian *ventricular* myocardium described in the present paper has however not been reported previously

It has been shown (Morad and Trautwein 1968 Antoni Jacob and Kaufmann 1969 Beeler and Reuter 1970 Tritthart *et al* 1973 b) that the duration of the action potential is one of the major factors in the control of the inotropic state of myocardial cells by governing the duration of both the calcium flux into the cell and the intracellular release of calcium that occurs during depolarization This concept is consistent with the finding that both the time to peak twitch tension and the action potential duration is reduced in the high HCO_3 solution This and other effects of the action potential shortening on the isometric twitch are discussed in more detail in the preceding paper (Johannsson and Nilsson 1975)

The shortening of the action potential observed in the present study in the high HCO_3 solution and in the propionate solution was unexpected for the following reasons 1 It has been observed that when chloride ions in the extracellular medium are replaced by larger and presumably less permeable anions the duration of the action potential increases (Hutter and Noble 1961 Carmeliet 1961 De Mello 1963) On this basis it has been surmised that a chloride current contributes to repolarization (Hutter and Noble 1961 Peper and Trautwein 1968) 2 Surface membranes of muscle cells are considered more permeable to chloride than to bicarbonate propionate and methylsulfate ions (Hutter and Noble 1961 De Mello 1963 Mainwood and Lucier 1972) When 47 mM of chloride are replaced by one of the above mentioned ions one would therefore expect a prolongation of the action potential because of diminished chloride current during repolarization The fact that bicarbonate and propionate caused a shortening of the action potential in the present study has therefore to be explained by something else than altered anionic currents An attractive possibility supported by findings in taenia coli (Casteels 1971) and in frog heart (Anderson and Foulks 1973) is that the potassium current during repolarization might be influenced by the extracellular anion composition

The authors wish to thank Professor K A P Edman for helpful discussions and for criticism of the manuscript and Miss Christina Olsson and Mrs Karin Johansson for excellent technical assistance The work was supported by grants from the Swedish Medical Research Council (project No 04X 184) and from the Medical Faculty University of Lund

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Acta physiol scand 1975 93 318–335

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Detection of Natural Complex Sounds by Cells in the Primary Auditory Cortex of the Cat

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Received 13 August 1974

Abstract

SOVIJÄRVI A R A *Detection of complex sounds by cells in the primary auditory cortex of the cat* Acta physiol scand 1975 93 318–335

The neural mechanisms involved in the detection of natural complex sounds were studied by recording single neuron responses from 132 cells in the primary auditory cortex of the cat The cats were paralyzed and under neuroleptanalgesia (NLA) The cells were first stimulated with pure tones the responses were then compared with those evoked by many different types of complex sounds most of which were animal vocalizations Per-stimulus-time (PST) histograms constructed from the responses to repetitive stimuli were compared with the corresponding sound spectrograms formed from the sounds used as stimuli Of 100 cells 68 per cent gave predictable responses to complex sounds on the basis of their responses to different pure tone frequencies In 32 per cent of the cells the responses were unpredictable Half of these cells did not react to pure tones at all but responded to one or more animal vocalizations or generator sounds with different patterns Some cells reacted to pure tones in quite a different way than to certain complex sounds e g with inhibition instead of excitation These results indicate that cells in the primary auditory cortex of the cat reacting in an unpredictable way to sounds with a complex structure have a more or less specialized function in detecting and analyzing natural and other complex sound patterns Cells reacting phasically to pure tones seem to be involved in the detection of transient sound elements

Key words

Cat Auditory cortex Unit activity sound discrimination–Complex sounds

Ablation studies have indicated that a special role of the auditory cortex is to encode complex auditory signals (Diamond and Neff 1957 Goldberg and Neff 1961 Kelly and Whitfield 1971 Cowey and Dewson 1972) Earlier single neuron recordings have also supported that theory The auditory cortex in the cat contains neurons which respond specifically to frequency modulated sounds (Whitfield and Evans 1965 Goldstein *et al* 1968 Suga 1968) and to certain complex tone combinations (Katsuki *et al* 1962 Feher and Whitfield 1966 Abeles and Goldstein 1972) as well as to combinations of frequency and amplitude modulation (Watanabe 1972) or to the "shape" of amplitude modulated noise (Swarbrick and Whitfield 1972) Moreover in monkeys a few cells have been shown to respond only to monkey vocalizations with specific acoustic properties but the responses

were not always dependent on simple acoustic features (Funkenstein *et al* 1971 Wollberg and Newman 1972 Newman and Wollberg 1973 Winter and Funkenstein 1973)

The papers cited above give rise to assume that some neurons in the auditory cortex could have specialized functional properties in detecting of complex sounds that are not simply frequency or amplitude modulated tones but have a more complex structure Therefore in this study a systematic search has been made in the primary auditory cortex of the cat for cells with properties that could be used for detection and analysis of different kinds of temporally complex sound patterns All neurons were first tested with pure tones and subsequently with a set of many different types of complex sounds most of which were animal vocalizations Also species-specific vocalizations were used The predictability of the responses of a neuron to complex sounds was determined from its response pattern and range to different pure tone frequencies

Because general anesthetics strongly depress the function of the auditory cortex (Erulkar *et al* 1956 Mountcastle *et al* 1957 Schlag and Brand 1958 Miller 1971 Noda and Adey 1973) neuroleptanalgesia (NLA) was used in this study (Sovijarvi and Saimio 1972)

Parts of the present results have been published earlier as congress abstracts (Sovijarvi 1972 Sovijarvi 1973)

Material and Methods

Mat i l

Single neuron recordings were made from 13 cells in the primary aud tory cortex of 18 adult domestic cats with weights ranging from 1 8 to 4 7 kg Only healthy cats responding behaviourally to sounds were used

Anesthes a

The form of anaesthesia was neuroleptanalgesia (NLA) combined with muscle relaxation (So ijärvi and Saimio 197) NLA was induced by inject ng fentanyl 0 0 5 mg kg, and dehydrobenzperidol 0 8 mg kg, intramuscularly (Thalamonal®) Muscle relaxat on was achieved with Galjam ne triethiodide (Flaxedil®) at on init al intramuscular dose of 4 mg/kg. Respirat on through an endotracheal tube was artif cial. As the experiments generally lasted 1?–15 h, addit onal doses of the drugs were necessary (Sovijärvi and Sa mio 197) A detailed d scussion of the methods of the anaesthesia used here is presented in an earl er related paper (Sov järvi and Hyvärinen 1974)

S gical procedures

Surgical procedures employed were the same as those described n the related paper (Sov järvi and Hyvä rinen 1974) The skull was opened over the primary auditory cortex of the right hemisphere with an electric drill A plexiglas cyl nder was fixed around the hole a hydraul c micromanipulator was attached to the cylinder which was filled with mineral o l forming thereby a closed chamber (Davies 1956)

Sou d g nerat on

Because a sound proof room with facil t es for microelectrode work was not a a lable at the time of th s st dy the experiments were carried out in a d mly lighted laboratory room ins de a Faraday cage which was kept as qu et as possible The sound pressure levels (SPLs) were measured w th a Bruel & Kjaer Prec s on Sound Lvel Meter ..03 supplied with an octa e f lter set. All the SPL measurements were referred to 0 000 dyn cm The level of background noise measured in the recording condit o s depended on the freque cy band in the bands from 63 to 500 Hz the SPL of noise aried between 5 and 35 dB and in the bands from 1 0 to 31 5 kHz between 14 and 21 dB No tape recordings were made d ring chance noises The standard SPL for pure tones was 80–85 dB and the peak SPLs of the n tural complex sounds varied from 64 dB to 95 dB When an acti e cells was found t was first tested w th pure tones in the range of 0 1 0 0 kHz generated with a Wa etek Wa eform Generator 155 All the st mulation sound

were fed to a Goodmans Twinaxiette 8 loudspeaker situated in the midline in front of the cat at a distance of 40 cm

The duration of the pure tones was 4 0 s it was triggered by a timing circuitry that also generated the trigger pulses used for computer analysis timed at 0 8 s before the tone For automatic analysis identical stimuli were repeated at a rate of 1 every 8 5 s 12–30 times in succession Some cells were also tested with complex sounds generated by a Wavetek Sweep Trigger Generator 114

The responses of the auditory cortical cells to complex sounds were compared with their responses to pure tones by offering a wide selection of natural complex sounds as stimuli These sounds were selected for their content of the various information bearing elements that are relevant in auditory communication (see Suga 1972) The types of natural complex sounds used can be classed as follows 1) harmonic like sounds with or without frequency modulation (e g vocalization of cats) 2) sounds containing short and successive complex sound elements with prominent frequency and amplitude modulation and comprising a wide frequency spectrum (e g the songs of the chaffinch and the nightingale) 3) sounds mainly consisting of noise elements (e g the call of a lemming) The time patterns of these sounds thus varied from tonal patterns to short rhythmic transients The natural sounds used were vocalizations of some birds and mammals which may be familiar to the cat The standard repertoire consisted of 17 taped vocalizations of 9 species cat (3 vocalizations) golden oriole guinea pig willow ptarmigan nightingale (2 vocalizations) chaffinch willow warbler lemming and barn swallow

All the examples of natural sounds had been taped with a Tandberg 4000 X tape recorder in the tape archives of the Finnish Broadcasting Company The standard stimulus tape was prepared in such a way that one channel consisted of 17 identical repetitions of 12 vocalizations and the other channel contained the trigger pulses for computer analysis These trigger pulses occurred 0 1–0 45 s before the beginning of the sound During renewals of the same sound the intervals mentioned above were naturally constant. The time intervals between repetitions of vocalization in different series varied from 4 to 6 s

The frequency spectra of the natural sounds used as stimuli were analyzed with the aid of a Voiceprint Sound Spectrograph 4691 A The shortest components of the vocalizations lasted about 0 msec (e g at the end of the song of the barn swallow) and the longest continuous sound the vocalization of a cat took 3 s The frequency area of the natural sounds was in general between 50 Hz and 8 0 kHz.

Some manually generated non specific odd sounds were also used as complex stimuli such as clapping and rubbing of hands jingling of keys and clinking of glass Speaking singing and whistling were sometimes useful as additional stimuli

Reco d ng and data colle tion

The extracellular recordings with platinum iridium microelectrodes with the use of a hydraulic micromanipulator the amplification and discrimination as well as the acoustic and visual monitoring of action potentials and the collection of data on magnetic tape were carried out as described in the earlier related paper (Sovijärvi and Hyvärinen 1974)

Data analysis

Some recordings were filmed from the oscilloscope with a kymograph camera (Grass C 4) Most of the neuronal responses were analyzed off line with a μ Linc computer programmed to construct per-stimulus time (PST) histograms of the responses to successive identical stimulus trials The duration of the samples for analysis was 4 0 s when taped natural sounds were used as stimuli and 8 0 s when pure tones and generator sounds were used The samples analyzed were thus longer than the stimuli and so the le el of spontaneous activity and any on and off responses could be seen The number of trials in the analyses varied from 8 to 3 The computed PST histograms were plotted on paper with a Calcomp 565 dig tal plotter Each bar in the x axes of the histograms represents 10 classes

The sound spectrograms (sonagrams) of the complex sounds were photographed on the same time scale as the PST histograms for detailed analysis of the timing of the responses in the PST histograms

S te of the elect ode

During each experiment the penetrat on points on the surface of the cortex were examined with a stereomicroscope and re-examined with reference to the average pattern of the sulci after decapitation of the animal and fixation of the brain tissue In 6 animals the areas from which recordings had been made were serially sectioned and stained with Nissl stain for nuclei From these sections the thickness of the cortex and the shape of the gyrus could be determined Comparison between the depth coordinates and the thickness of the cortex establ shed that the recordings were made from neurons of the primary aud tory cortex A map of the penetration points is shown in Fig 1

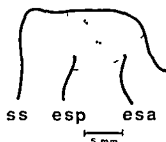


Fig. 1 Site of the electrode tracks on a standard diagram of the auditory cortex in the right hemisphere The interrupted line indicates the boundary of the primary auditory cortex (AI) according to Woolsey (1960) (ss) suprasylvian sulcus (esp) posterior ectosylvian sulcus (esa) anterior ectosylvian sulcus

Results

Recordings were made from 132 cells in the primary auditory cortex of the cat Not all these cells could be tested with the complete sound stimulation programme which lasted more than one hour because the activity of some cells could not be recorded for long enough Thus 125 cells were adequately tested with pure tones and so could be classified according to their response patterns In 100 of these cells the response properties to pure tones and complex sounds were compared

General features of neuronal activity in the auditory cortex

In this material only 4 cells showed no activity without acoustic stimuli the others were firing spontaneously Among 132 cells examined 11 (8%) did not react to any of the acoustic stimuli used although they showed spontaneous activity Cells with high spon

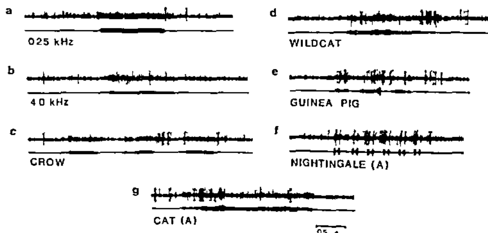


Fig. — Photographic records of the responses of cell 16-1 to selected pure tones (a–b) and to complex animal sounds (c–g). The upper traces indicate the action potentials of the neuron and the lower traces the oscillograms of the simultaneous stimulus sounds This figure shows examples of both inhibitory ( ) and excitatory (b) responses to pure tones and predictable responses to complex sound patterns

TABLE 1 Classification of the response patterns of auditory cortical cells tested with pure tones

		Number of cells	Per cent
Total number of cells tested		125	100
I Excitatory responses		35	28
A Sustained excitation	32		25
B Partial or adaptive excitation	2		2
C "On" response and excitation	1		1
D "Off" response and excitation	0		0
E "On-off" response and excitation	0		0
II Inhibitory responses		33	27
A Sustained inhibition	9		7
B Partial or adaptive inhibition	4		3
C "On" response and inhibition	2		[illegible]
D "Off" response and inhibition	12		10
E "On-off" response and inhibition	6		5
III Phasic responses only		8	6
A "On" response	1		1
B "Off" response	3		2
C "On-off" response	4		3
IV More than one response type in different frequency areas		21	17
A Excitatory and inhibitory response areas	13		10
B Different combinations of "on" "off" and inhibitory responses in different frequency areas	8		7
V No response to pure tones		28	[illegible]

taneous activity gave in general better responses to sounds than those with low spontaneous activity

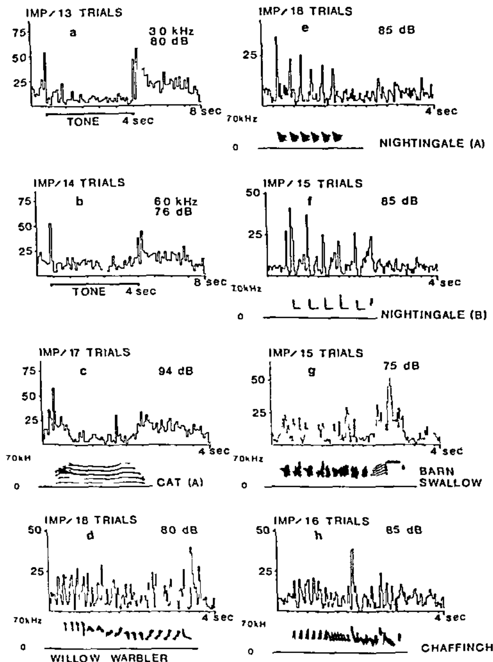
The lability of the response patterns was a feature common to many cells but other cells however gave stable responses to different types of sound stimuli

Many cells tested generated more impulses during the initial part of long stimuli than later thus showing adaptation to the stimulus Habituation of the responses to successive presentations of the same stimulus was also common Almost invariably however "habituated" cell responded readily to a novel stimulus

Responses to pure tones

Response ranges to pure tones were mapped at a sound pressure level of 80-85 dB from 0 1 to 20 0 KHz (see Methods) 15% of the cells tested the largest group had narrow

Fig. 3 Per-stimulus-time (PST) histograms computed from the responses of cell 40-1 7 to successive trials of pure tones (*a* and *b*) and to complex sounds (*c h*) The response pattern consisting of an "on" component followed by inhibition and an "off" response (*a* and *b*) is reflected in a predictable way (exact time locking) in the response patterns to complex sounds consisting of transient elements (*d h*) In this and the following figures the y axes of the histograms indicate the number of neuronal impulses in each time interval class summed from numerous trials the number of which is indicated above each histogram The x axes indicate time the bars represent 10 classes The pure tone stimuli are presented as a bar and the frequency of the tone is indicated above each histogram The frequency spectra of the complex stimulus sounds are shown as sonagrams on the same time scale as the corresponding histograms just above them. The bars in the sonagrams indicate the frequency in kHz The peak SPL of each stimulus is shown above each histogram in dB


UNIT 40-1-7
IMP/13 TRIALS
a
30 kHz
80 dB
TONE 4 sec
8 sec
IMP/18 TRIALS
e
85 dB
4 sec
70kHz
0
NIGHTINGALE (A)
IMP/14 TRIALS
b
60 kHz
76 dB
TONE 4 sec
8 sec
IMP/15 TRIALS
f
85 dB
4 sec
70kHz
0
NIGHTINGALE (B)
IMP/17 TRIALS
c
94 dB
4 sec
70kH
0
CAT (A)
IMP/15 TRIALS
g
75 dB
4 sec
70kHz
0
BARN SWALLOW
IMP/18 TRIALS
d
80 dB
4 sec
70kHz
0
WILLOW WARBLER
IMP/16 TRIALS
h
85 dB
4 sec
70kH
0
CHAFFINCH

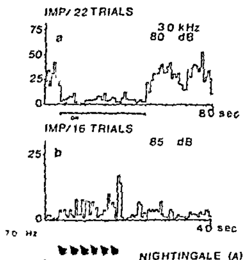


Fig. 4 Two PST histograms computed from the evoked activity of cell 40-1 3 In *a* a 3 0 kHz tone evoked sustained inhibition but in *b* a complex sound pattern the song of nightingale A evoked excitatory responses

response ranges Cells with response ranges up to 7 5 octaves were also found 19 cells (22%) had 2, and 2 cells 3 separate response ranges (multiranged cells) Most of the multi ranged cells had both inhibitory and excitatory response ranges Eight cells also had ranges within which the responses were purely phasic The threshold curves of some cells were measured in 4 expts Both narrowly and broadly tuned cells were recorded as well as cells with curves of multiple minima

A wide variety of response patterns to pure tones was found However almost every cell could be placed on the basis of its response pattern in one of he major groups in Table I Of the 125 cells classified 37 (30%) had phasic on and off components in their response patterns Phasic responses were much more commonly given by cells that showed inhibition in their responses than by excited cells only one excited cell showed a response pattern which included phasic elements Examples of the response types are shown in Fig. 2-4

Predictable responses to complex sounds

68 per cent of the cells tested responded to complex sounds in a way which could be predicted from their responses to pure tones (Table II) Predicted sustained (non phasic) responses excitatory or inhibitory to tones and complex sounds were obtained from 33 per cent of the cells Phasic responses to tones and predicted time locked responses to transient complex sounds were found in 24 per cent of the cells Of the 100 cells tested 11 per cent gave no response to pure tones or to the complex sounds used Some examples are presented on the following pages

Cell 16-1 2 (Fig. 2) responded to a 0.25 kHz tone with inhibition and a slight offset burst (*a*) but had an excitatory response range from 0 5 kHz to 18 0 kHz (*b*) Utterances of a crow consisting of harmonic elements and noise in an area of 0 6-6 0 kHz, evoked inhibition and "off" responses best seen during the second and third utterances in c

TABLE II Classification of auditory cortical cells by their responses to complex sounds and pure tones

		Number of cells		Per cent
Total number of cells tested			100	100
I Responses to complex sounds were predictable from the responses to pure tones			68	68
A *Sustained (non phasic) responses to pure tones and complex sounds*		33		33
B Phasic responses to pure tones and time locked responses to transient complex sounds		24		24
C No response to pure tones or complex sounds		11		11
II Responses to complex sounds were not predictable from the responses to pure tones			32	32
A No response to pure tones but a response to specific types of complex sounds		17		17
a Specific natural complex sounds from tape	10			
b Odd complex sounds only	6			
c FM sweep and odd complex sounds	1			
B No response to complex sounds but a response to pure tones		4		4
C Different types of responses to pure tones and to specific types of complex sounds		11		11

Thus in this case the inhibitory effect of the low frequency sound components was stronger than the excitatory effect of the higher frequencies On the other hand the vocalization of a wildcat (*d*) consisting of harmonic components between 0 2 kHz and 5 1 kHz evoked excitation not inhibition But in the terminal part of the sound where the sound spectrogram revealed noise elements the function of the cell was inhibited This inhibition may have been induced by the noise components in the sound although these were not within the inhibitory range of the cell as they were in the sounds of the crow (*c*) However inhibition only occurred when some sound components were within the inhibitory range The upward sweeping harmonic sounds of a guinea pig (*e*) which contained frequencies only within the excitatory range of the unit evoked intense excitation with weak habituation during the third utterance The song of a nightingale (*f*) also evoked excitation which was not precisely time locked as was that of cells giving phasic responses The vocalization of a cat (*g*) produced moderate excitation as could be predicted no elements of this sound lay within the inhibitory area of the cell

Cells reacting with on or off components to pure tone frequencies gave usually exactly timed response patterns The best time locking in the responses to transient sounds was observed in cells which gave both on and off responses to pure tones as the responses of cell 40-1 7 in Fig 3 The response range of this cell was 0 8–18 0 kHz and the responses to pure tones showed inhibition between on and off components (*a* and *b*) The cat vocalization shown in *c* evoked an on response inhibition and a small off response A short excitation in the rising FM part before the end of the sound was found too Time locked responses were evoked by all short sound components as seen in *d–h* The shorter the sound component and the steeper the slope of the frequency the stronger were the responses of the cell For instance the first 40-ms sound component in the song of nightingale

B in *f* evoked saharp excitation peak in the histogram which was not seen in the response pattern of the off' type cells to the same stimulus The best activation was evoked by the 20 ms click elements at the end of the song of the barn swallow (*g*) This cell also gave better timed responses to the song of the chaffinch (*h*) than off" type cells

Unpredictable responses to complex sounds

Of the 100 cells tested (Table II) 32 responded to complex sounds in a way which was not predicatable from their responses to pure tones Of these cells 17 did not show any response to pure tones in the frequency area used (0 1–20 0 kHz) at SPLs of 80–85 dB 10 cells responded readily to one or several of the taped natural complex sounds but not at all to pure tones 6 cells reacted only to "odd" complex sounds such as clapping of hands jingling of keys etc One cell responded both to FM sweeps and to odd complex sounds but did not react to pure tones used

Fig. 5 relates to cell 34-1-6 which did not respond to pure tones at all (*a*) Neither did the momentary complex sounds of nightingale songs or harmonic vocalizations of cats elicit any clear response However the third utterance in the song of a golden oriole (*b*) which consists of falling harmonic elements evoked excitation The song of a willow warbler elicited some good responses locked to the rhythm of the song, as shown in *c* This cell did not respond to FM sweeps but jingling of keys caused rhythmic excitation The spontaneous activity of this cell decreased during the test period as seen in the histograms (from *a* to *e*)

Another example of a "complex cell is shown in Fig. 6 This cell did not react to any of the pure tone frequencies used an example is shown in *a* A rising falling FM sweep in the range between 0 5 and 7 0 kHz with a duration of 4 s did not evoke any response either as seen in *b* Nor did the natural complex sounds in the standard stimulation programme drive this cell its spontaneous activity was unaltered by exposure to the harmonic sounds transients and noise elements in these sounds For instance in *d* the call of a lemming had no effect on the form of the PST histogram Yet a complex sound produced by the Wavetek generators evoked the vigorous excitation shown in *c* This "gurgling sound included repetitive steeply rising FM components and falling FM parts in terraces as seen in the sonagram Furthermore these complex components were combined with three pure tone frequencies of 0 1 0 9 and 1 8 kHz, which when used alone did not evoke any response

Four cells responded to certain pure tones but not to any of the complex sounds used. One of these cells is shown in Fig. 7 This cell responded to pure tones in the range of 1 5–2.7 kHz with sustained excitation that was not, however very intense A 2 5 kHz tone which was the characteristic frequency of the cell evoked the excitation shown in *c* But the complex sounds used caused no response although most of them consisted of frequency elements lying within the response range of the cell (*a* and *b*)

Of these 100 cells 11 gave unpredictable response patterns to complex sounds compared with the patterns of the pure tone responses Some cells had only excitatory response ranges but reacted with inhibition to certain complex sounds On the other hand some cells gave only inhibitory responses to pure tones but responded with excitation to certain

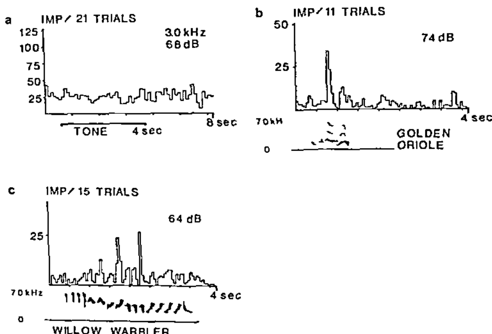


Fig 5 PST histograms computed from the activity of cell 34-1-6 In *a* the cell appears unresponsive to a 3 0 kHz tone stimulus. In *b* and *c* the histograms illustrate excitation in response to the songs of a gold-n oriole and a willow warbler These sounds also contain frequency elements around 3 0 kHz as seen from the sonagrams

sound patterns Some complex sounds evoked phasic on and off' responses which were not evoked by any of the pure tones and vice versa

Cell 40-1 3 (Fig 4) responded with sustained inhibition in the 1 0–11 0 kHz range The response to the characteristic inhibitory frequency of 3 0 kHz is shown in *a* However only a few complex sounds gave any response at all and inhibition was not observed in the responses to complex sounds The songs of the chaffinch and nightingale A caused excitation and an off response in an unpredictable way An example is shown in *b* In this response pattern however no exact time locking to transient sounds was seen As the sonagram shows *the pattern of this nightingale song consists of frequencies which are all within the* inhibitory response range of the cell including 3 0 kHz The level of spontaneous activity of this cell became lower during the recording period (from *a* to *b*)

Discussion

Anesthesia

General anesthetics profoundly depress the cellular function of the cerebral cortex (Mount castle *et al* 1957 Noda and Adey 1973) Hence they also decrease or abolish the spon

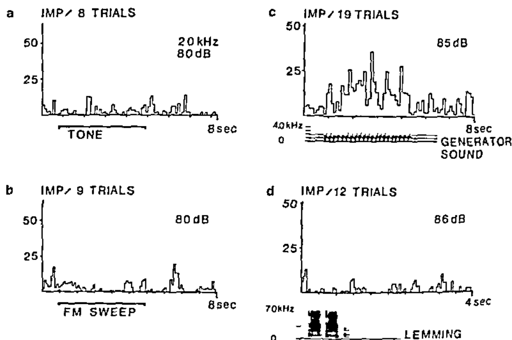


Fig. 6 PST histograms from cell 36-2 10 In *a* a 2 0 kHz tone does not evoke any response In *b* a rising falling FM sweep in the range of 0 5–7 0 kHz did not alter the histogram. In *c* clear excitation is evident in the response to a complex generator sound But, as seen in *d* transient and noisy elements forming the utterance of a lemming did not cause any response

taneous activity of the auditory cortical cells and substantially reduce their responsiveness to acoustic stimuli (Erulkar *et al* 1956 Katsuki *et al* 1959 Sovijärvi and Saimo 1972) For these reasons general anesthesia was avoided and replaced by neuroleptanalgesia combined with muscle relaxation This kind of combined anesthesia does not significantly alter the electrical function of the auditory cortex in the cat (Sovijarvi and Saimo 1972) and it was needed for eliminating the painful and disagreeable stimuli produced by the preliminary surgical measures and for keeping the test animal immovable

Acoustic conditions

The experiments were performed in an ordinary laboratory room designed for microelectrode work with background noise at a higher level than it would be in a sound proof room (see Methods) The SPLs of the stimuli varied from 65 dB to 95 dB exceeding the back ground noise level by 50–75 dB Thus the signal to-noise ratio was sufficient for auditory discrimination But as the auditory system has been demonstrated to adapt to stationary noise (see Kärjä 1968) presumably lack of a sound proof room does not invalidate the results Moreover the background noise level during the experiments was to some extent comparable to the noise level in the natural environment of the cat

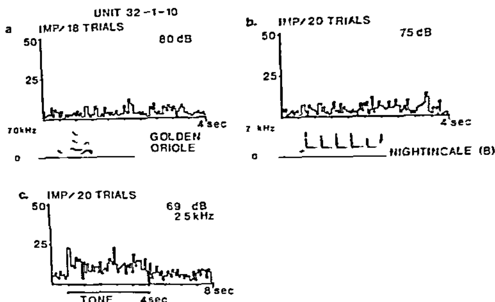


Fig. 7 PST histograms of the responses of cell 32-1 10 to sound stimuli, showing sustained excitation to a 2.5 kHz pure tone (the characteristic frequency) (*c*), but no responses to complex sounds which sweep over the characteristic frequency (*a* and *b*)

Responses of auditory cortical cells to pure tones

Seventy-eight per cent of the spontaneously active cells tested in these experiments responded to pure tones (see Table I) This proportion of tone-sensitive cells was about the same as found by Abeles and Goldstein (1972) in the primary auditory cortex of unanesthetized and paralyzed cats On the other hand Evans and Whitfield (1964) reported that only 54 per cent of the cells in the A I on unanesthetized and unrestrained cats responded to pure tones and Bogdanski and Galambos (1960) obtained a value of 70 per cent The difference between the present results and those mentioned in the two latter papers could conceivably be due to the differences in the analyzing systems Phasic "on" and "off" responses and weaker sustained responses, especially when inhibitory may sometimes pass unnoticed unless dot displays or per-stimulus time (PST) histograms are constructed as was done here

The distribution of the different types of response patterns to pure tones (Table I) displayed some interesting features Firstly cells giving excitatory and inhibitory responses were almost equally common (28 and 27 per cent, respectively) Secondly phasic "on" and "off" responses were mostly combined with inhibition only one cell showed phasic patterns with excitation Thirdly as many as 30 per cent of the cells tested displayed phasic features in their response patterns

These results confirm previous findings that phasic responses are quite common in the auditory cortex both in cats (Bogdanski and Galambos 1960 Gerstein and Kiang 1964) and in monkeys (Katsuki *et al* 1962, Funkenstein *et al* 1971) Inhibition or suppression of spontaneous activity by tone stimuli has been reported before Bogdanski and Galambos

(1960) found 15 per cent and Evans and Whitfield (1964) 10 per cent But in the present study a much higher proportion of cells responding in this way was found 27 per cent As before the discrepancy is probably due to the difference in the sensitivity of the analyzing system The stimulation methods and the criteria of classification may of course have contributed to the differences in the results

The fact that inhibitory and phasic responses were almost as common as excitatory responses in the auditory cortical cells implies the existence of multiple excitatory inhibitory interactions The importance of such interactions in complex sound analysis and in sound localization is obvious

Previous workers have shown that some auditory cortical cells may have different types of response patterns in different frequency areas and at different SPLs (Goldstein *et al* 1968 Abeles and Goldstein 1972) In the present study 17 per cent of the cells tested showed differences in their response patterns when the frequency was different but the SPL was the same (Table I) The same cell might give both excitatory and inhibitory responses in *different ranges sometimes widely separated from each other (Fig 2) In some* cells however the change of the response pattern was gradual especially when phasic responses were combined with inhibition The cells giving the same or different types of response in several separate response ranges (multiranged cells) all had at least one narrow response range The same phenomenon was noted by Suga (1964 1965 a b) in the cells of the inferior colliculus and the auditory cortex of echo locating bats Many cells had only one response range narrow or wide excitatory inhibitory or purely phasic Such response ranges have frequently been observed before (Evans and Whitfield 1964 Oonishi and Katsuki 1965 Abeles and Goldstein 1970)

Cells with multiple response ranges of different widths each range tending to have an individual response pattern presumably have some connection with the analysis of complex sounds

The function of phasic cells in the detection of transient sound patterns

As already *mentioned many of the cells studied showed* phasic responses to pure tones mostly combined with inhibition When these phasic cells were tested with complex sounds differing in time structure but having some or all of their sound energy within the response range of the cell the following observations were made Sounds closely resembling pure tones *e g* harmonic sounds with a vocalic character emitted by cats excited the cells only at the onset or offset of the stimulus sound but caused inhibition or no response at all during the other parts of the sound the pattern thus resembling the response to pure tones When some frequency modulation or noise components were included in the sound the responses to such components were excitatory Especially when stimulated with sounds composed of a succession of transient elements or clicks such as occur in the songs of nightingales the phasic cells reacted as a rule in an exactly time locked fashion The best time locking to transient sounds was seen in cells responding to pure tones with an on-off pattern (Fig 3) In this respect these phasic cells resemble those called lockers by de Ribaupierre *et al* (1972)

On the other hand cells giving sustained excitation to pure tones did not respond

synchronously to repetitive transient sound complexes the timing of their responses was comparatively inaccurate De Ribaupierre et al place cells of this kind either in a category they call groupers characterized by loose synchrony or in a group called special response patterns in which there is no time locking to repetitive clicks

Such results indicate that the phasic cells in the auditory cortex are involved in detecting the onset and offset of acoustic signals and play a specific role in detecting repetitive momentary sound complexes

Predictable time locked responses to transient sounds in phasic cells were quite common being found in 24 per cent of the cells studied (Table II)

Complex sound detection by cells giving sustained (non phasic) responses to pure tones

a *Predictable function* A high proportion of the cells (33°) responding to pure tones with sustained excitation or inhibition without phasic components reacted predictably to complex sounds (Table II) Thus cells reacting with sustained excitation to pure tones in a certain frequency range or ranges were driven by all the types of complex sounds used provided that at least some of the sound components were within the response ranges of the cell Cells of this kind which could be called general responders or simple cells usually had high spontaneous activity This finding is consonant with a report of Wollberg and Newman (1972) who found good responsiveness to all kinds of sound stimuli applied to monkey auditory cortical cells which usually have high spontaneous activity

In the same way inhibitory cells were found which gave predictable sustained inhibition in response to all complex sounds containing elements within the response range of the cell However most inhibitory cells also had phasic components in their response patterns (Table I) and some responded in a selective way to complex sounds Hence the proportion of the sustained inhibitory response cells that reacted in a predictable way to complex sounds was low

Some cells had both excitatory and inhibitory response ranges without clear phasic components in their response patterns The detection of complex sounds by these cells was somewhat more complicated If the sound components were all within a single range the cell gave responses that were typical of this range But if the sound consisted of frequency components in both the inhibitory and the excitatory ranges of the cell the type of response depended on how large a proportion of the sound energy was within the inhibitory or excitatory range of the cell or on other structural features of the sound (see Fig 2)

Presumably such a predictably responding cell is capable of nonselective detection of those frequency components of a complex sound that are within the frequency range or ranges of the cell Impulse trains from simple cells of this kind might then serve as input to cells in the auditory cortex which have a more complex function in the analysis of acoustic information

b *Unpredictable function* This study revealed some cells in which spontaneous activity was not affected by any of the complex sound patterns used but which were driven by pure tones in a certain frequency area (Table II and *e g* Fig 7) An interesting point is that no inhibitory range could be found in these cortical cells However inhibitory mechanisms activated by complex properties of the sounds must in some way be involved in un

predictable responses of this kind possibly at subcortical levels This suggestion is supported by Suga s (1968) finding that an excitatory response of a cell to the characteristic frequency could be inhibited by another simultaneous tone the frequency of which was outside the response range of the cell An excitatory inhibitory interaction was seen at the cortical level in cells which responded with sustained excitation to the pure tones in a frequency range but were inhibited by certain complex sound patterns consisting of frequency ele ments within the excitatory range Sometimes the situation was reversed pure tone stimuli caused inhibition and some complex sounds evoked excitatory responses in the same cell (Fig 4) For some cells the repetition rate of the transient sound complexes was the factor on which the unpredictable inhibition depended for instance hand-clapping at a rapid rate sometimes caused total inhibition but time locked bursts were generated when the rate was slow This finding indicates the existence of delayed or conditioned inhibitory mechanisms of the kind recently demonstrated by Abeles and Goldstein (1972) in the auditory cortical cells of the cat

Existence of speciali ed cells for detection of complex sound patterns

In the present study 8 per cent of the 132 cells tested although firing spontaneously did not react in any way to the acoustic stimuli used they responded neither to pure tones nor to complex sounds Compared with earlier studies the proportion of acoustically silent cells was lower in the present investigation Evans and Whitfield (1964) found that 23 per cent and Bogdanski and Galambos (1960) that 18 per cent of the cells in the auditory cortex were unresponsive to acoustic stimuli in unanesthetized and unrestrained cats In these investigations however complex acoustic stimuli were used only occasionally thus only a few cells responding specifically to complex sounds could be found On the other hand Goldstein *et al* (1968) who used only swept tones as complex sound stimuli found less than 5 per cent of silent cells in the primary auditory cortex of cats immobilized with gallamine triethiodide It seems probable that this muscle relaxant when given without any anesthetics reduces the proportion of silent cells by its activating effect on the auditory system (Halpern and Black 1967)

In the present study 17 per cent of the cells in the primary auditory cortex responded only to certain complex sounds but not to any of the pure tone frequencies used (Table II) Most of these functionally complex cells responded to one or more taped animal vocalizations which were usually acoustically much alike But sometimes the effective sounds that gave specific responses had somewhat different frequency spectra (see Fig 5) No cells responding only to species-specific vocalizations were found when the cells were tested with cat vocalizations of three different kinds The situation might have been different if behaving animals had been used These findings could be compared with the recent results of Funkenstein *et al* (1971) Wollberg and Newman (1972) Newman and Wollberg (1973) and Winter and Funkenstein (1973) who studied the responses of monkey auditory cortical cells to species specific vocalization They found that 2–3 per cent of the cells gave responses only to some acoustically very similar monkey calls usually consisting of prominent FM components or a noise spectrum But they also found some cells which responded exclu sively to one or other of two closely similar monkey calls although responding to certain

other types of calls also Although these authors did not use vocalizations of other species and did not test every cell with pure tones these findings seem to be in according with the present results The present study likewise revealed cells driven only by certain odd complex sounds (Table II) like jingling of keys or clapping of hands or in a few cases a rapidly varying complex sound from a sound generator (Fig 6) These cells were unaffected by complex sounds included in the standard set of taped animal vocalizations

The cells specialized for detection of complex sounds differed in their responses to the same complex sound In general however the effective sound patterns included short steep FM components The responses of these cells were all excitatory no cells responding only to complex sounds reacted with inhibition

The present finding that as many as 32 per cent of the cells in the auditory cortex gave unpredictable responses to complex sounds was not unexpected Almost the same percentage of unpredictable responses (37%) was reported by Winter and Funkenstein (1973) in their study of the responses of auditory cortical cells in the monkey to species specific vocalization The features that might have triggered the function of this kind of specialized cell were amply represented in the sounds used in this study: variable types of frequency and amplitude modulations and tone combinations were often represented in one animal vocalization All of these factors separately have been shown to be cues for the specific reaction of cells in the auditory cortex (Whitfield and Evans 1965 Goldstein *et al* 1968 Suga 1965 Oonishi and Katsuki 1965 Feher and Whitfield 1966 Abeles and Goldstein 1972 Watanabe 1972) It is also likely that not only the structure of the sound but also the rate of repetition might be a critical factor for the function of a cell in the sound detection process

Suggested function of the auditory cortical cells in detection of complex sounds

The present results indicate that many of the cells in the primary auditory cortex of the cat contribute in a specific way to the processing and detection of temporally complex acoustic signals However the degree of functional complexity of these cells seems to vary About two thirds of the cells tested in the present study responded in a predictable way to complex sound patterns Such cells may be concerned with detecting the type of a sound pattern conveying the information to higher-order neurons that then discriminate the individual sound patterns Thus for instance cells responding phasically to pure tones were also mostly time locked to transient sound complexes The cells that responded in an unpredictable way to complex sounds may function as higher-order neurons with different degrees of specialization the neurons with the most selective ability to identify sounds seem to be those which respond only to one or more complex sounds which may be entirely different in structure The meaning of the sound probably plays some role in such selective responsiveness It seems likely that in other auditory areas there are more neurons which play a specialized role in the detection of complex sound patterns receiving inputs from the primary cortical area as well as from lower levels of the auditory pathway

Here it has been shown that a certain population of cells in the primary auditory cortex has a special role in the detection of complex sounds As reported in the earlier related paper (Sovijärvi and Hyvärinen 1974) some of these same cells which responded in a time locked

fashion to sound transients also reacted to directional movements of the sound source Thus a neuron in the primary auditory cortex is not restricted to functioning in one complex way

This study has been supported by a grant from the Finnish Cultural Foundation
This paper is based on the doctoral thesis of Dr Sovijärvi entitled "Single Neuron Responses to Complex and Moving Sounds in the Primary Auditory Cortex of the Cat" Institute of Physiology University of Helsinki Helsinki 1973

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Acta physiol scand 1975 93 336–344

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Effects of Myocardial Infarction on Adrenergic Nerves of the Rat Heart Muscle, a Histochemical Study

By

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Received 13 August 1974

Abstract

AHONEN A M HARKÖNEN J JUNTUNEN M KORMANO and A PENTTILA *Effects of myocardial infarction on adrenergic nerves of the rat heart muscle a histochemical study* Acta physiol scand 1975 *93* 336–344

The anterior branch of the left coronary artery of the rat heart was ligated and the effects of anoxia on adrenergic nerves were studied histochemically using formaldehyde induced fluorescence for localization of norepinephrine Greenish catecholamine fluorescence was associated in the normal or infarcted myocardium only with adrenergic nerves Constant but not prominent changes were seen in adrenergic nerve fibers 2 or 4 h after ligation of the coronary artery the number of delicate adrenergic fibers was reduced and some diffusion of the histochemical reaction was seen in small areas of the infarcted myocardium. Strong effects of anoxia were seen 8 h or more after ligation of the coronary artery These were characterized by a prominent diffusion of the histochemical reaction and gradual disappearance of adrenergic structures in 2 to 4 days of anoxia During the healing phase the appearance of numerous adrenergic nerve fibers was closely connected with the ingrowth of a new vascular bed into the infarction area The observations indicate that adrenergic nervous structures of the myocardium resist the effects of anoxia which may indicate the viability of sympathetic nerves in the infarcted area during long periods of anoxia

The high concentration of norepinephrine its active removal from arterial circulation and the ability to synthesize norepinephrine from precursors are characteristic of the mammalian heart muscle (Potter *et al* 1965) Histochemical (Angelakos *et al* 1963 1966 1969) and radioautographic (Potter *et al* 1965) studies suggest that norepinephrine is associated with sympathetic nerves of the myocardium and that the number of nerves varies greatly in different regions of the heart (Angelakos *et al* 1969) Observations on human and experimental materials suggest that plasma level and urinary excretion of norepinephrine increase following myocardial infarction (Gazes *et al* 1959 Lammerant *et al* 1966 Richardson *et al* 1960 Richardson 1963 Shahab *et al* 1969) Based on chemical determinations (Russell *et al* 1961 Shahab and Wollenberger 1967) it has been assumed that norepinephrine is released from its myocardial adrenergic storage sites and that this may be one important

reason for increased plasma levels of norepinephrine Histochemical studies however have suggested that adrenergic nerves of the rat myocardium are resistant against the post mortem effects of anoxia and acidosis (Penttila 1971) In view of these observations we studied histochemically the response of adrenergic nerves to anoxia in experimental infarct of the rat myocardium using the specific histochemical method in the localization of catecholamines

Material and Methods

The material comprised 60 female rats including 10 sham-operated control animals The rats received tap water ad libitum but no food for 24 h before and 12 h after the operation The operations were performed in aether anesthesia and 0 1 ml of atropine sulphate (Orion Oy Helsinki) was given to the animals 1-3 h before the operation Hair was cut and the skin was sterilized with 70 aqueous alcohol The left fourth intercostal space and the anterior wall of the pericardial sac were dissected The descending branch of the left coronary artery was readily visible on the anterior surface of the left ventricle and it was ligated just distal to the apex of the left auricle with one silk ligature (Ethicon type 000) using an atraumatic needle Manual compression of the heart was avoided and the operations were performed as carefully as possible in order to avoid venous stasis and damaging the heart muscle A small area of necrosis was later seen around the ligat on site and the infarcted anterolateral wall of the left ventricle The operation wound was closed tightly using several silk sutures going around the 4th and 5th ribs and a drainage tube provided with constant suction was left in the thora ic cavity for 5-10 min Sometimes spontaneous breathing ceased during operat on, but was readily reco ered when artificial respiration was used The sham-operated rats were treated and operated identically but the descending branch of the left coronary artery was not ligated The mortal ty of the rats was about 15% and it was of about the same order in the sham-operated and experiment rats

The rats were killed from 10 min to 4 weeks after ligat on of the coronary artery and the myocardial samples of experiment and sham-operated rats were taken as rap dly as possible During the post infarction period of 8 h or more the infarction area was ident f ed macroscopically by its pale color relaxed myocardium and hemorrhages During the early post infarction period (10 min-4 h) the whole heart was cut horizontally into two pieces The proximal part was applied for histochemical phosphorylase reaction to localize the ischemic zone Pieces for fluorescence studies were taken from the distal part One larger ca 0 5 mm thick sample that contained the whole anterolateral wall of the left ventricle and the foremost portion of the interventricular wall was used for gross orientation In addition 5-10 small p eces were taken from various areas of the ischemic myocardium of the l ft ventricle The cases w th normal histochemical phosphorylase re ction were d scharged

The samples for phosphorylase reaction were rinsed in saline at 0 C placed on a t ssue holder co ered with 10 aqu ous solution of gelat ne frozen in l quid nitrogen and 10 µm sections were cut on a cryostat The histochem cal method developed by Takeuchi *et al* (1955) was used The pieces for fluorescence microscopy were taken as soon as possible frozen in isopentane precooled with l quid nitrogen and stored in liquid nitrogen until freeze-dried The methods used for histochemical demonstration of catecholamines followed the princ ples developed by Eränkö (1955 1964) Fal k (196) and Falck and Owman (1965) The spec mens were dried in vacuo at -40 C for 1-2 days slowly warmed up in vacuo and exposed to formaldehyde vapor deri ed from paraformaldehyde at 60 hum dity The pieces were embedded in paraffin in vacuo and stud ed immed ately The autofluorescence was studied in the specimens treated as above but without paraformaldehyde treatment. The sod um borohydride test was used in the ident f ca t on of adrenergic nerves during the repair phase (Corrodi *et al* 1964)

Results

During the early period of anoxia the use of histochemical reaction was necessary for demonstration of ischemia in the myocardium In about 5 per cent of the cases the operation was not successful which further emphasized the use of methods other than gross

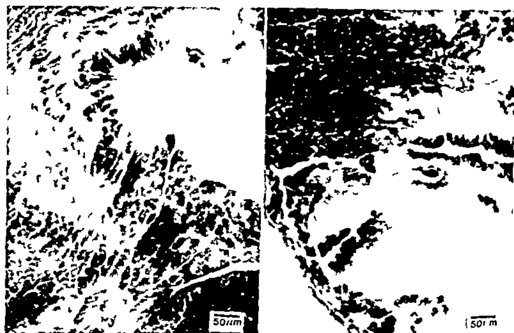


Fig 1 Phosphorylase reaction at the border zone of the anticipated infarction area of the anterior wall in the left ventricle of the rat 5 min after ligation of the descending branch of the left coronary artery The pale zone indicates the loss of activity due to anoxia

Fig. 2 Phosphorylase reaction 10 min after operation Pale zone indicates loss of phosphorylase activity

examination for early localization of the infarcted area The effects of anoxia were already demonstrable 5–10 min after ligation of the coronary artery by reduced myocardial phosphorylase activity (Fig 1 and 2) This early change is consistent with several earlier reports on anoxic heart muscle of the rat (*e g* Bajusz and Jasmin 1963 Jääskelainen 1966) An uneven distribution of phosphorylase activity was characteristic of the infarcted area up to 1–2 h of anoxia followed later by a total loss of histochemically demonstrable activity

The anterolateral wall of the left ventricle of the normal rat heart contained a moderate

Figs 3–11 Formaldehyde induced fluorescence in the anterior wall of the left ventricle of the rat heart The thickness of the sections was 10–15 μm Histochemical phosphorylase reaction was used as an indicator of the presence of anoxia in adjacent specimens

Fig. 3 Myocardium 30 min after the sham-operation Several delicate fluorescent adrenergic structures with varicosities are seen Many of the tortuous fibers are cut longitudinally The more compact fluorescent structures are mast cells

Fig. 4 Myocardium 30 min after the operation No essential effects of anoxia on fluorescence properties of adrenergic nerves can be seen Note the dense patterns of adrenergic nerve bundles around a larger arterial branch

Fig. 5 Myocardium 60 min after the operation The intact endocardial layer is more clearly seen by its stronger autofluorescence Gross sections of adrenergic nerve fibers are quite evenly distributed in the infarcted and non-infarcted myocardium

Fig. 6 and 7 Myocardium 8 h after the operation Some strongly fluorescent and distinct adrenergic nerves are seen in better preserved myocardium (Fig 6) whereas the adrenergic nerves in the more severely injured center of the infarction zone exhibited mostly a prominent diffusion of the reaction product (Fig 7).

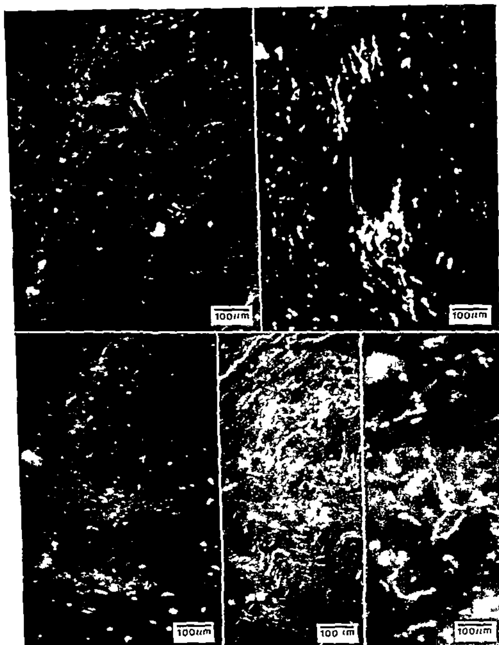


Fig 3–7

number of delicate and a few coarse nerve bundles (Fig 3) All adrenergic fibers exhibited a strong and distinct greenish fluorescence readily identifiable on the practically non fluorescent background The pattern of fluorescent adrenergic structures displayed was dependent on the niveau used for cutting the sections Longer tortuous fibers with varicosities were seen in sections cut longitudinally in the direction of the muscle fibers (Fig 3 and 4) The patterns of adrenergic nerves were similar in normal and sham operated rats killed at varying intervals after operation No fluorescent catecholamine-containing cells were seen in the infarct area or in the controls Several mast cells localized mostly in the vicinity of vascular channels were identified by their yellowish and strong 5 hydroxytryptamine fluorescence (Fig 3 and 9 a)

It was characteristic of adren rgic nerves of the myocardium that they were resistant to the effects of anoxia in the infarcted area as estimated on the basis of their fluorescence properties No essential changes could be identified in catecholamine fluorescence patterns of the anterolateral wall of left ventricle during 10 to 60 min of anoxia (Fig 4 and 5) Neither were the changes 2 or 4 h after ligation of the coronary artery prominent Small areas with diffusion of the fluorescent compound associated with nerve fibers were seen This was in patchy small areas also associated with a reduction in the number of delicate adrenergic nerves Catecholamine fluorescence of coarser nerve bundles was similar to that in the controls and the varicosities of delicate fibers retained their intense fluorescence for longer periods than the fibers

A strong but uneven effect of anoxia on the fluorescence patterns of adrenergic nerves was seen in 8 hour-old myocardial infarcts (Fig. 6 and 7) The number of fluorescent adrenergic structures was greatly reduced and the disappearance of adrenergic nerves was mostly associated with more advanced myocardial deterioration in the infarct zone A prominent diffusion of fluorescent compound often seen around larger nerve bundles in the core of myocardial infarction (Fig 7) was another characteristic phenomenon in deterioration of adrenergic nerves 8 h or more after ligation of the coronary artery the intensity of the autofluorescence of the injured myocardium was moderate or strong so that it was difficult to identify catecholamine fluorescence on the yellow greenish autofluorescent background making the use of the sodium borohydride test necessary

Progression of deterioration of adrenergic structures due to anoxia and other effects following ligation of the coronary artery was also more prominent in 12 24- and 48 hour-old infarcts (Fig. 8 9 a and b) Large non fluorescent areas were found in 2-day-old infarcts and only occasional small fluorescent adrenergic nerve structures were seen in the vicinity of larger vascular channels or at the endocardial border of the infarcted zone but they were no longer visible in 4-day-old infarcts

During the repair phase from 1 to 4 weeks after ligation of the coronary artery there were increasing numbers of adrenergic nerves at the epicardial border and lateral areas of the infarcted myocardium (Fig 10 and 11) Later adrenergic nerves were seen in relation to the repair time everywhere in the healing myocardial zone mostly associated with new vascular channels The repair tissue was also characterized by several types of strongly yellowish or greenish autofluorescent cells and debris making the use of the sodium borohydride specificity test necessary for identification of adrenergic nerves (Fig. 10 and 11)

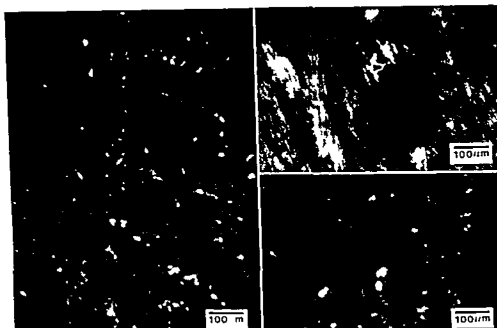


Fig 8 Myocardium 1 day after the operation Some fragments of adrenergic nerves are seen in the center of the infarcted area

Fig 9 a and b Myocardium 2 days after the operation Occasional adrenergic nerves were seen around vascular channels whereas quite many intact looking mast cells were seen in the infarcted myocardium

Discussion

The predominant catecholamine in the heart of several laboratory animals including the rat is norepinephrine (Angelakos *et al* 1969) Evidence available on the sensitivity of the histochemical procedure employed for demonstration of catecholamines in tissues (Falck 1962) including the heart muscle (Angelakos *et al* 1969) suggests that under optimal conditions practically all adrenergic structures are demonstrable In the present study the distinct catecholamine fluorescence of the normal heart muscle was found to be associated only with varicose nervous fibers which is consistent with earlier reports on the heart muscle of several species (Angelakos *et al* 1963 1966 1969 Schiebler and Heene *1968*) *In view* of these observations the histochemically demonstrated catecholamine fluorescence associated with adrenergic nerve fibers can be practically regarded as specific for norepinephrine This also supports the histochemical method (Falck and Owman 1965) and the specificity test (Corrodi *et al* 1964) used in norepinephrine demonstration

The present histochemical observations indicated that norepinephrine associated with adrenergic nerves in the infarcted myocardium was resistant against the effects of anoxia and other possible effects of ligation of the coronary artery as estimated on the basis of catecholamine fluorescence properties No effects in fluorescence were seen at 1 h small effects at 2 and 4 and strong effects at 8 h of anoxia or later Observations on the rat musc

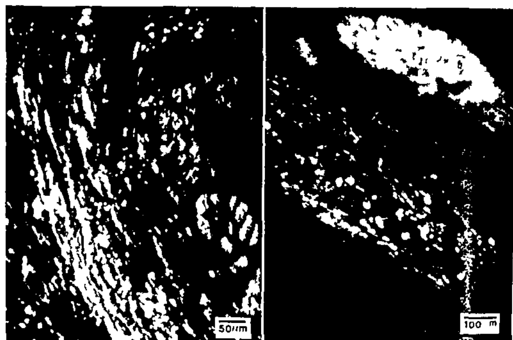


Fig 10 Myocardium 2 weeks after ligation of the coronary artery Identification of adrenergic nerves was based on the use of the sodium borohydride test because many types of structures of the repair tissue and debris exhibited a strong autofluorescence

Fig 11 Myocardium 4 weeks after the operation Strongly autofluorescent cells and fibers in addition to catecholamine-fluorescent nerve fibers are characteristic of the scar tissue replacing the myocardium

have been quite similar in relation to time of post mortem anoxia (Penttilä 1971) Because the concentration of norepinephrine of the heart muscle has proved to be in rough agreement with the density of adrenergic fibers in several species (Klouda 1963 Angelakos *et al* 1969) the present histochemical observations lend support to chemical observations on experimental myocardial infarct of the dog (Russell *et al* 1961 Mathes and Gudbjarnason 1971) which indicated that the level of norepinephrine declined prominently in the infarct zone 1–2 days following ligation of the coronary artery Total disappearance of catechol amine-containing structures in the infarct 4 days after occlusion of the coronary artery as stated in this study is also in agreement with chemical data on canine heart muscle (Mathes and Gudbjarnason 1971) The present results do not lend any support to the observations of a rapid decline of norepinephrine in the rat heart due to anoxia of a few minutes as stated chemically by Shahab and Wollenberger (1967) which may be the reason for the different type of experimental design they used Neovascularization of the infarcted zone was accompanied again by a few histochemically demonstrable adrenergic nerves

The resistance of adrenergic nerves to anoxia is emphasized when compared with other myocardial changes due to anoxia in similar experimental conditions used in the present study (Penttila *et al* 1974) High-energy phosphate compounds declined in concentration in the infarct zone immediately after ligation of the coronary artery The release of nore

pinephrine from adrenergic nerves was not associated with this although it is assumed that this amine in sympathetic nerve endings is stored as ATP norepinephrine complex (Euler *et al* 1963 Iversen 1967) The first irreversible electron microscopical changes in the myocardial cells were seen 30 min after ligation of the coronary artery accompanied by a total loss of glycogen and prominent enzyme histochemical changes which clearly preceded the response of adrenergic nerves to anoxia The histochemically stated survival of adrenergic fibers in the infarction zone is an obvious indication of their viability and possibly also of the presence of some functional activity during long periods of anoxia One reason for their survival may be their close association with vascular channels because the presence of some perfusion has been demonstrated using roentgenographic methods in the infarct zone of the rat heart (Penttila *et al* 1974) The present observations indicate that the initial depletion of norepinephrine from adrenergic nerves of the infarct zone is obviously associated with partial destruction of neural elements demonstrable by diffusion of the fluorescent compound and occasional fragmentation of nerve fibers followed by total loss

This study was supported by grants from Finnish Heart Association the National Research Council for Medical Sciences of Finland and the Sigrid Juselius Foundation

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Control of the Neurotoxicity of 6-Hydroxydopamine by Intraneuronal Noradrenaline in Rat Iris

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Received 22 August 1974

Abstract

SACHS CH G JONSSON R HEIKKILA and G COHEN *Control of the neurotoxicity of 6 hydroxydopamine by intraneuronal noradrenaline in rat iris* Acta physiol scand 1975 *93* 345–351

In vitro studies with the neurotoxic compounds 6-hydroxydopamine (6-OH–DA) and 6-aminodopamine (6-A–DA) showed that noradrenaline (NA) markedly inhibited the autooxidation of 6-OH–DA but not of 6 A–DA *In vivo* studies of the adrenergic nerves in rat iris showed that the neurotoxic potency of 6-OH–DA but not 6-A–DA was increased after NA depletion by α methyl *p* tyrosine methylester (H44 68) Neurotoxicity was evaluated by measuring the associated decrease in ³H-NA uptake Intraocular injection of NA counteracted the degenerative action of 6-OH–DA in both untreated and H44/68 pretreated rats Intraocular NA did not interfere with the neurotoxicity of 6-A DA Additionally octopamine did not affect the rate of autooxidation nor the neurotoxic potency of 6-OH–DA or 6-A–DA Control experiments with H 6-OH–DA showed that the intraneuronal NA levels did not significantly affect the intraneuronal accumulation of 6-OH DA The parallelism between the *in vitro* results on autooxidation and *in vivo* data on neurotoxicity makes it appear that the neurotoxic potency of 6-OH–DA and 6-A–DA is closely associated with their rates of autooxidation The control of the degenerative action of 6-OH DA by intraneuronal NA may be mediated via reaction of NA with radicals formed from oxygen during autooxidation of 6-OH DA

Key word 6-Hydroxydopamine 6-aminodopamine adrenergic nerves noradrenaline degeneration

6-Hydroxydopamine (6-OH DA) is well known for its specific neurotoxic action on catecholamine neurons (Tranzer and Thoenen 1967 see symposium volume edited by Malmfors and Thoenen 1971) The cytotoxicity of 6-OH DA is considered to be associated with its ease of autooxidation whereas the specificity of action has been shown to be related to the uptake and accumulation of 6-OH DA by a transport mechanism specific for catecholamine neurons (Thoenen and Tranzer 1968 Jonsson and Sachs 1970 Ljungdahl *et al* 1971) Although the exact molecular mechanisms leading to neuronal damage and subsequent degeneration are unknown it has been shown in *in vitro* experiments that during the autooxidation of 6-OH DA several potentially toxic products are formed These include 6-OH DA

quinones hydrogen peroxide (H_2O_2) and several free radicals all of which have been suggested to play causative roles in the degeneration process (Saner and Thoenen 1971 Heikkila and Cohen 1971 1972 1973 Cohen and Heikkila 1974) Two radicals which have been detected during the autooxidation of 6-OH DA are the superoxide (O_2^-) and the hydroxyl (OH) radical There is evidence that these radicals can be trapped by catecholamines (Misra and Fridovich 1972 Cohen and Heikkila 1974) Of special interest is the fact that the superoxide radical promotes the over all oxidation rate of 6-OH DA (Heikkila and Cohen 1973) Since the degenerative effect of 6-OH DA is potentiated after depletion of the noradrenaline (NA) content in adrenergic nerves (Jonsson and Sachs 1973 a) the question can be raised Does the trapping of free radicals by intraneuronal catecholamines control the neurotoxic potency of 6-OH DA? The present study was undertaken to investigate this possibility The results obtained strongly indicate that NA can reduce the rate of autooxidation of 6-OH DA as well as the degree of neurotoxicity on sympathetic adrenergic nerves For comparison with 6-OH DA identical experiments were carried out with 6-aminodopamine (6-A DA) a compound previously shown to possess neurotoxic properties on catecholamine neurons similar to that of 6-OH DA (Heikkila *et al* 1973 Jonsson and Sachs 1973 b)

Materials and Methods

Autooxidation studies in vitro The rate of oxidation of 6-OH-DA and 6-A-DA in Krebs Ringer phosphate buffer pH 7 4 was investigated by monitoring the rate of increase in absorbance at 490 nm (rate of formation of quinone products) and by measuring the O consumption (see Heikkila and Cohen 1973) The reactions were carried out at +37 C The colorimetric studies were performed on a Gilford Model 350 spectrophotometer with a flow through cuvette (Gilford Instruments Oberlin Ohio) The studies on oxygen consumption were carried out with a Clark oxygen electrode (Yellow Springs Instruments Yellow Springs, Ohio) connected to a Honeywell Electronik 19 Recorder (Honeywell Instruments Minneapolis, Minn) The chemicals used were NA HCl octopamine HCl (Winthrop) 6-OH DA HBr (Regis) and 6-A-DA 2HCl (gift of Dr E Engelhardt Merck Sharpe and Dohme West Point, Pa and Dr R N Adams Lawrence Kansas) Stock solutions of 6-OH-DA and 6-A-DA were prepared in nitrogen sparged distilled water

Neurotoxicity studies in vivo Untreated rats (male Sprague Dawley 150-?00 g) and rats treated with α meth l *p*-tyrosine methylester (H44 68 Hässle AB Goteborg 500 mg/kg *i p* 16 h) were injected with 50 ng NA HCl (Sigma) or octopamine HCl (Sigma) in 5 μl isotonic NaCl into the anterior chamber of the right eye with a microsyringe (Hamilton) The left eye serving as control received an equal volume of saline 6-OH DA (5 mg kg Hässle AB Göteborg) or 6-A-DA (10 mg/kg) was injected *i v* 60 min later in 0 ml saline and the animals were then killed after 4 h by cervical dislocation under light chloroform anesthesia Thereafter the irides were rapidly dissected out and incubated *in vitro* at +37 C for 30 min in Krebs Ringer bicarbonate buffer pH 7 4 containing 10^{-7} M ^3H-NA (spec activity 5-10 Ci mmole Radiochemical Centre Amersham) After rinsing for 10 min in ice-cold buffer the irides were dissolved in 0 5 ml tissue solubilizer (Soluene-TM[1] Packard Instr) and 10 ml toluene phosphor was added Total radioactivity was determined by liquid scintillation spectrometry Under these experimental conditions 90 or more of the radioactivity taken up and accumulated in the irides represents unchanged ^3H NA (Jonsson *et al* 1969)

In control experiments rats were injected *i v* with ^3H-NA HCl (25 μCi in 0 ml saline) or ^3H-6-OH DA HBr (1 or 5 mg kg in 0 ml saline spec activity 1 0 mCi mmole a gift from Dr A Saner F Hoffmann La Roche Basle) Total radioactivity taken up and retained by the irides was determined as described above For the evaluation of extra neuronal uptake accumulation of radioactivity after ^3H-6-OH DA administration, one group of animals was treated *i p* with 25 mg kg of desipramine (DMI Pertofran Geigy) 30 min before the H-6-OH DA injection

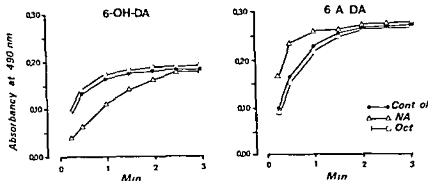


Fig 1 Eff ct of NA (10^{-3} M) and octopamine (10^{-4} M) on the rate of formation of quinoidal products during the autooxidation of 6-OH-DA (10^{-4} M) or 6-A-DA (10^{-4} M) The reaction was carried out at +37 C with constant agitation in Krebs-Ringer phosphate buffer at pH 7 4 Results are the mean of tri plicate determinations which agreed within 5 This experiment was repeated several times with similar results

Results

Effects of NA on the autooxidation of 6 OH DA and 6 A DA

Fig 1 shows the rate of autooxidation of 10^{-4} M 6-OH DA or 10^{-4} M 6-A DA as followed colorimetrically by measuring the increase in absorbance at 490 nm (formation of quinoidal products see Heikkila and Cohen 1973) Octopamine had no measurable effect on the rate of autooxidation of either 6-OH DA or 6-A DA NA at 10 ³ M did affect the rate of autooxidation of 6-OH DA or 6-A DA but in opposite directions there was inhibition of the rate for 6-OH DA but stimulation of the rate for 6-A DA The effects of various concentrations of NA on the autooxidation of 6-OH DA and 6-A DA are shown in Fig 2 Data are expressed in terms of the initial rate of reaction which was taken as the increment in absorbance at 490 nm in the first 15 s of reaction In the 6-OH DA system there was 34 inhibition by 10^{-4} M NA and this increased to 53 and 56 at 10⁻ M or 10 ⁸ M ($p<0.01$) respectively In the 6-A DA system NA had no effect at 10 M or 10 ³ M however at 10 M NA a stimulation of 66 % ($p<0.01$) was observed With either 6-OH DA or 6-A DA octopamine was without effect at 10^{-3} M (Fig 2) 10^{-3} M or 10^{-4} M (data

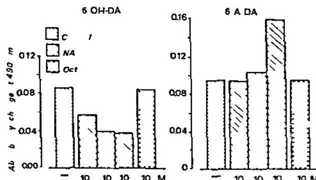


Fig Effect of NA (10^{-4} M 10^{-3} M and 10^{-8} M) or octopamine (10⁻ M) on the init al rate of autooxidat on of 6-OH-DA (10^{-4} M) or 6-A DA (10^{-4} M) Cond t ons are the same as in Fig 1 The initial rate is given by the increase in absorba ce at 15 s

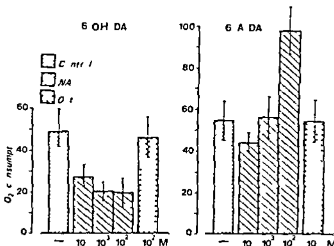


Fig 3 Effect of NA or octopamine on the initial rate of oxygen consumption by 6-OH-DA or 6-A DA The reaction system is the same as in Fig 1 A Clark oxygen electrode was used to measure the disappearance of oxygen from the solution in a closed 1 ml system (n = 3 to 6) Data are nmoles O_2/min ± S D

not shown) The effect of NA or octopamine on the autooxidation of 6-OH DA or 6-A DA as studied with an oxygen electrode (oxygen consumption) was also investigated (Fig 3) In this system 10^{-3} M octopamine was again without effect on the rate of autooxidation of 6 OH DA as well as 6-A DA NA produced effects parallel to those observed in the colorimetric system Thus NA at 10^{-5} M inhibited oxygen consumption by 44% and the effect was increased to 58% at 10^{-4} M and 60% at 10^{-3} M ($p < 0.01$) In the 6-A DA system there was once again a stimulatory effect by 10^{-3} M NA (76% $p < 0.01$) which was not observed at either 10^{-5} M or 10^{-4} M NA In other experiments similar results on rates of autooxidation were obtained when dopamine was substituted for NA or when phenylephrine was substituted for octopamine

Effects of NA on the neurotoxicity of 6 OH DA and 6 A DA

One of the very early signs (within 1 h) of the degeneration process induced by 6-OH DA in sympathetic adrenergic nerves is severe damage of the NA uptake mechanism at the axonal membrane the so-called membrane pump (Jonsson and Sachs 1970 1972) There is complete destruction of a number of nerves depending on the dose used while the remaining nerves are left with apparently intact NA uptake storage mechanisms Therefore it is possible to quantitatively evaluate the neurotoxic effects of 6-OH DA by measuring the ^{3}H NA uptake *in vitro*

Using this technique (*in vitro* uptake of ^{3}H NA) we found that the intraocular injection of NA significantly counteracted the neurotoxic action of 6-OH DA in irides from both untreated rats and rats pretreated with H44/68 (Fig 4 left side) The intraocular injection of NA by itself was without effect on the uptake of ^{3}H NA With 6-A DA on the other hand no significant decrease in neuronal damage was seen after NA was administered intraocularly (Fig 4 right side) It was also observed that H44/68 pretreatment potentiated the reduction in ^{3}H NA uptake by 6-OH DA but not 6-A DA From Fig 5 it can be seen that intraocular injection of octopamine was ineffective in modifying the 6-OH DA changes in ^{3}H NA uptake both in untreated rats and rats pretreated with H44/68

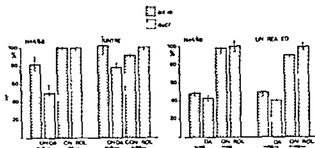
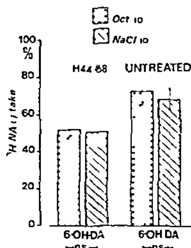


Fig 4 Effect of NA injected intraocularly (*i o*) on the *in vitro* uptake of ^{3}H-NA in rat iris after 6-OH-DA or 6-A-DA administration Rats pretreated with H44/68 (500 mg/kg *i p* 16 h) and untreated control rats were used. The intraocular injection of NA (50 ng/5 μl right eye) and saline (5 μl left eye) was carried out 60 min before the 6-OH DA (5 mg/kg *i v*) or 6-A-DA (10 mg/kg *i v*) administration and the rats were killed 4 h thereafter The irides were then isolated for *in vitro* incubation in ^{3}H-NA (10⁻ M 30 min) and the radioactivity taken up and retained was determined. Each column represents the mean ± S E. of 11-15 determinations and is expressed as a percentage of the untreated and the H44/68 pretreated control respectively H-NA uptake accumulation in controls were in H44 68 pretreated 16 3±0 97 nCi/iris and in untreated 17 2±0 89 nCi/iris Student s t test was used for the statistical evaluation (* = 0 05 > p > 0 01 ns = not significant, p > 0 05)

In order to evaluate whether or not the protective action of intraocular NA was related to an interference of the uptake accumulation of 6-OH DA *in vivo* 2 kinds of expts were done First untreated rats received a unilateral injection of NA intraocularly as in previous experiments and 60 min later ^{3}H NA (25 μCi) was administered systemically (*i v*) The rats were killed 15 min after the ^{3}H NA injection and the radioactivity taken up and retained in the irides was determined There was no significant difference between the two sides (saline 2 6 ± 0 11 nCi/iris NA injected 2 9 ± 0 18 nCi/iris n = 4) This indicated that the intraocular injection of NA did not interfere with the membrane pump uptake mechanism of the adrenergic nerves Second rats pretreated with H44/68 were injected with NA intraocularly on one side and 60 min later H-6-OH DA (1 or 5 mg/kg *i v*) was administered As seen from Fig 6 there was no difference in radioactivity present in the irides between the 2 sides after either dose of ^{3}H 6-OH DA One group of rats was pretreated with the potent membrane pump blocker DMI in order to distinguish neuronal uptake (blocked by DMI) from extraneuronal uptake The data of Fig 6 show that the neuronal accumulation of radioactivity after ^{3}H-6-OH DA was unchanged after intraocular injection of NA

Discussion

The present experiments were undertaken to clarify the mechanism whereby H44 [illegible] depletor by tyrosine hydroxylase inhibition) sensitizes sympathetic adrenergic [illegible] son and Sachs 1973 a) to the degenerative action of 6-OH DA The results of [illegible] ments showed that NA markedly inhibited the rate of autooxidation of 6-OH [illegible] 6-A DA (Fig 1 2 3) These data correlated well with the *in vivo* results on the [illegible] of these compounds (Fig 4) It was thus observed that NA depletion [illegible] potentiated the 6-OH DA induced damage (reduction in ^{3}H NA uptake [illegible] hand, exogenous administration of NA leading to increased intraneuronal [illegible] acted the degenerative action of 6-OH DA both in untreated rats a [illegible]



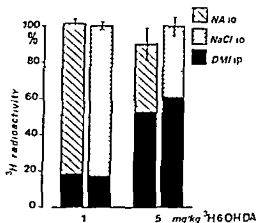


Fig. 5 Effect of octopamine (Oct., 50 ng 5 µl) injected intraocularly on the *in vitro* uptake of ³H NA (10^{-7} M 30 min) in rat iris after 6-OH-DA (5 mg/kg i.v.) administration to rats pretreated with H44 68 and to untreated control rats. The experimental conditions are the same as in Fig. 4 Each column represents the mean ± S E. of 9–11 determinations and is expressed as a percentage of the H44 68 or untreated control respectively

Fig. 6 Effect of NA injected intraocularly on the uptake of radioactivity in iris after i v administration of ³H-6-OH-DA to rats pretreated with H44 68 (500 mg/kg i.p 16 h) NA (50 ng 5 µl) was injected into the right and saline (5 µl) into the left eye 60 min later ³H-6-OH-DA (1 or 5 mg/kg) was injected i v One group of rats was treated with DMI (25 mg kg i p) 30 min before ³H-6-OH DA 15 min after the ³H-6-OH DA inject on the irides were isolated for the radioactivity determination. Each column represents the mean ± S E. of 4–8 determinations and is expressed as a percentage of the saline treated control.

treatment Neither of these treatments (H44/68 or intraocular NA) had any significant effect on the neurotoxicity of 6-A DA In addition intraocular administration of octopamine a substance known to be taken up and accumulated in adrenergic nerves (Snyder *et al* 1964 Kopin *et al* 1964) was found ineffective in modifying the neurotoxicity of 6-OH DA or 6-A DA Consistently octopamine was also without effect on the autooxidation of 6-OH DA or 6-A DA *in vitro* This correlation between the *in vitro* and *in vivo* data thus favours the view that neurotoxic potency is closely associated with the rate of autooxidation of 6-OH DA and 6-A DA Since exogenous administration of NA did not significantly affect the accumulation of ³H-6-OH DA in the adrenergic nerves it is clear that the quantitatively different neurotoxic effects seen after increasing or decreasing the NA levels were not related to a varying accumulation of 6-OH DA intraneuronally

It was noted that NA enhanced the rate of autooxidation of 6-A DA *in vitro* (Fig. 1 2, 3), whereas no potentiation of the neurotoxicity was seen *in vivo* after NA loading (Fig 4). The potentiation of the 6-A DA autooxidation was however only found at very high NA concentrations (10^{-4} M) which may be difficult to reach *in vivo* during the conditions used.

The present data show that NA inhibits the rate of autooxidation of 6-OH DA and also its neurotoxic action on adrenergic nerves Octopamine can not substitute for NA in inhibiting either the rate of autooxidation or the neurotoxic action of 6-OH DA The most attractive explanation for the inhibitory effect of NA on neurotoxicity is that it reacts with radicals derived from oxygen (Cohen and Heikkila 1974) during the autooxidation of 6-OH DA Although the present data do not provide conclusive results as to the agent or

agents initiating the degenerative process we suggest that the inhibitory effect of NA is mediated by removal of the superoxide radical (cf Misra and Fridovich 1972) which has been shown to control the overall oxidation rate of 6-OH DA (Heikkila and Cohen 1973) Thus, the intraneuronal transmitter content within CA neurons can be a significant factor in modifying the neurotoxic action of 6-OH DA This may explain, at least partly some of the differences in sensitivity to 6-OH DA seen in different CA neurons and systems

The present study has been supported by grants from the Swedish Medical Research Council (04X 3881) Karolinska Institutet, M Bergvalls Stiftelse and t e United States Public Health Service (NS-05184) The skilful technical assistance of Eva Lindqvist, Bodil Flock and Felicitas Cabbat is gratefully acknowledged

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Acta physiol scand 1975 93 352–363
From the Department of Physiology and Biophysics University of Lund Sweden

The Action of Dibutyryl Adenosine-3 5′-Cyclic Monophosphoric Acid and Theophylline on the Isolated Cat Parotid Gland

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Abstract

FRITZ M E and W GRAMPP *The action of dibutyryl adenosine 3 5 cyclic monophosphoric acid and theophylline on the isolated cat parotid gland* Acta physiol scand 1975 93 352–363

In the isolated cat parotid gland intraarterially applied dibutyryl cyclic AMP (db-cAMP) (1 0 mM) produced a slow but often maintained salivary flow db-cAMP was also able to potentiate secretion evoked by supramaximal doses of intraarterially applied acetylcholine Similar secretory effects were obtained also after intraarterial application of theophylline (1 0 mM) The secretory responses due to db-cAMP or theophylline were unaccompanied by measurable acinar membrane potential changes and stayed unchanged after cutting the parasympathetic innervation of the gland and after blocking both cholinergic and β-adrenergic receptors with atropine (10^{-7} M) and with D-(−)-N isopropyl p-nitrophenol-ethanolamine (INPEA) (10^{-4} M) respectively The possibility of the existence of an acinar adenylate cyclase system functionally linked to the β-adrenergic membrane receptor is discussed

Key words Parotid gland cyclic AMP theophylline salivary secretion secretory potentials

Previous studies have demonstrated that adenosine 3 5 -cyclic monophosphoric acid (cAMP) or some of its derivatives can elicit fluid and/or enzyme secretion in exocrine glands (Scratcherd and Case 1973) Similar effects have also been obtained with drugs such as theophylline which are known to inhibit the cAMP degrading action of intracellular phosphodiesterase

Data have been presented so far which suggest that cAMP acts as an intracellular mediator for secretin in the cat pancreas (Case and Scratcherd 1972 a) for adrenaline in rat rabbit and dog salivary glands (Batzri and Selinger 1973 Kojima Ikeda and Tsujimoto 1971 Martinez and Martinez 1972 a and 1972 b) and for 5 hydroxytryptamine in insect salivary glands (Prince and Berridge 1973) The present investigation was performed to assess the involvement of cAMP as a possible link in the process of excitation secretion coupling in the cat parotid gland The cat parotid gland was chosen particularly with

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respect to recent observations that nervous control of the gland is exerted not only by parasympathetic fibres but also although to a lesser and more variable extent by sympathetic fibres acting on β adrenergic membrane receptors (Emmelin Schneyer and Schneyer 1973 Ekstrom and Emmelin 1974)

Methods

26 cats (1 9 to 2 9 kg) were used in the present study The animals were fasted 18-24 h before the experiments Anesthesia was induced with Pentothalsodium (50 mg/kg I P) and later maintained with the same drug in dosages of 10 mg/kg administered through a femoral vein catheter During the operat on the animals were artificially respirated through a tracheal cannula and their blood pressure monitored through a femoral artery catheter

For electrophysiological recording from the parotid or submaxillary gland *in situ* the glands were surgically exposed and decapsulated over a small area (approximately 3 × 3 mm) Subsequently they were tied along their edges to the animal frame to minimize disturbances from respiratory movements

For experimentat on on the isolated parotid gland th gland and its salivary duct were surgically exposed (*f* Fritz 1977) After this the arterial supply of the gland was dissected according to the description by Mia and Sis (1970) whereby all branches of the external carotid artery except those leading to the gland w re ligated Likewise the venous drainage of the gland was isolated by ligating all contributories to the external jugular vein except those lea ing the parotid gland Subsequently both the external carotid artery and the external jugular ve n were cannulated with polyethylene tubing, and artificial perfus on of the gland was initiated The perfusion fluid had the following composition (in m moles/l) NaCl 140 KCl 4 0 $CaCl_2$ 1 5 MgCl 1 0 Na_2HPO_4 0 6 and glucose 5.5 it was fully equilibrated with oxygen and had a pH of 7 4 Perfusion of the gland was performed at room temperature at a rate of 4 ml m n which corresponds to normal blood flow through the stimulated cat parotid gland (Burgen and Emmelin 1961) the perfusion pressure under these conditions varied between 60 and 1 0 mm Hg in different animals. After assuring a successful perfusion and enous collection the salivary duct was cannulated with a fine glass catheter which was connected to polyethylene tubing leading to a drop counter Finally after checking that the gland responded properly to intraarterial injections of acetylcholine a small area of the gland was decapsulated and the animal killed by an over-dose of the anesthetic in order to el minate any remain ng blood circulation through the gland

In one cat denervat on of the parotid gland was carried out n ne days before the actual exper ment The denerv tion was performed according to Burgen (1964) by sectioning the auriculotemporal nerve at the tubercle of the mandible

Acetylcholine (ACh) was used to elic t sal vary secretion This drug was injected into the perfus ng solution be means of a micrometer dri en syringe in amounts of 3 to 10 μg dissol ed in 3 to 10 μl of fluid The injection was carried out through a three way stopcock (opened only for injections) mounted close to the gland so that the time interval between injection and electrophysiological response did not exceed 4-6 s

The flow of sali a was measured by use of a photo-electric drop counter This instrument was calibrated to measure flow rates of up to about 15 drops min which was well abo e the maximal amo nts of secretion in the present preparation

Electrophysiological examinat on of the gland was carried out using standard intracellular record ng techniques The m croelectrodes were filled with e ther 3 M KCl or M K-citrate and had resistances rang ng from 15 to 50 megohms For impalement of cells the electrode attached to the electromet r ampl fier probe was advanced in μm steps with a stepmotor dri en mic oman pulator During ad ance me t of the electrode its t p was agitated for periods of about 1 ms at a repetition rate of 2 s^{-1} by means of a p ezo-electric dri e fitted to the amplifier probe (Grampp and Sjölin 1973) Us ng this de ce a greater number of cells were successfully impaled than otherwise A cell was considered to be successfully m aled when is membrane potential after penetrat on rema ned stable for at least 30 s

Drugs we applied to th gland b add ng th m to the perfusing solut on Adenosine 3 5 -cycl c mo ophosphoric acid (cAMP) and N O^2-d butyryl adenosine-3 5 -cycl c monophosphori acid (db-cAMP) (both Sigma Chem cal Company St Lou s Mo U S A) were g en in 0 1 1 0 mM and theophyll ne (BDH Chem cals Ltd Poole England) in 0 1-5 0 mM concentrat on At op ne sulphate w s added to the

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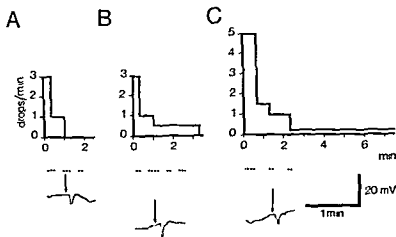


Fig 1 Upper row salivary secret on in isolated artificially perfused parotid glands evoked by 10 μg ACh under control cond tions (A) by 5 μg ACh in the presence of 1 0 mM db-cAMP (B) and by 10 μg ACh in the presence of 1 0 mM theophylline (C) Lower row membrane and secretory potentials recorded from acinar cells during secretion as shown in the upper row The interrupted lines indicate zero potential The arrows mark the time of ACh injection

secreted during a period of 1–3 min the maximal rate of flow was of the order of 3 drops/min (Fig 1 A) Electrophysiologically impaled units responded to single injections of ACh with so called secretory potentials i e 6–10 s long hyperpolarizations with peak amplitudes of 3 to 15 mV (*cf* Fig 1 A lower row) In the present preparation electrophysiological and secretory responses were thus of the same magnitude as those elicited in the cat parotid gland by repetitive supramaximal (for flow) stimulation of the auriculotemporal nerve (Fritz and Botelho 1969 b)

Effects of dibutyryl cyclic AMP and theophylline on salivary secretion and membrane potentials in the isolated preparation

Addition of 1 0 mM db-cAMP to the perfusate of the isolated cat parotid gland consistently evoked a small flow (1 drop every 2–3 min) of saliva (Fig 2 B) Often this salivary flow was sustained throughout the presence of the cyclic AMP but in a number of preparations it persisted only for a limited time (8–10 min) initially during the drug exposure

A similar but possibly somewhat less pronounced effect was obtained after addition of 1 0 mM theophylline to the perfusate (Fig 2 C)

In contrast to its dibutyryl derivative the parent substance cAMP in two cats produced no salivary flow unless theophylline was also added to the perfusate However since by applying the two agents simultaneously it was possible to obscure the effects of either the use of cAMP was abandoned in the further course of this study

In addition to evoking secretory responses as described above both db-cAMP and theophylline were able to potentiate the secretory effects of single supramaximal doses of ACh This potentiation consisted of an augmentation of the initial rate of secretion as well as of a prolongation of the response to about twice its normal duration (Fig 2 B and 2 C)

In the case of theophylline this potentiating effect was found to be dose dependent As

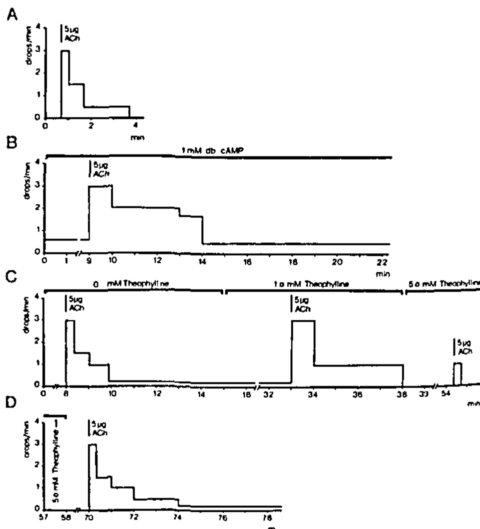


Fig Salivary secretion in an isolated artificially perfused parotid gland A secretory response evoked by 5 µg ACh (time of injection marked by arrow) under control conditions B secretory response induced by 1 0 mM db-cAMP and by 5 µg ACh in the continued presence of 1 0 mM db-cAMP C secretory responses induced by various concentrations of theophylline (as indicated by horizontal brackets) and by 5 µg ACh in the presence of various theophylline concentrations D secretory response evoked by 5 µg ACh administered 12 min after perfusion with 5 0 mM theophylline

shown in Fig 2 C 0 1 mM theophylline produced only a minimal residual flow of saliva but no augmentation of the initial response to a supramaximal dose of ACh Such an augmentation as well as a prolongation of the secretory response was however apparent with 1 0 mM theophylline In 5 0 mM concentration theophylline had an inhibitory effect both on sustained salivary secretion previously induced by theophylline in lower concentrations and on ACh evoked secretory responses (Fig 2 C) This inhibitory effect was reversible on re institution of control perfusion when the re appearance of potentiated responses to ACh injections indicated a gradual wash-out of the high theophylline concentration (Fig 2 D)

TABLE I Resting membrane potentials measured in the isolated cat parotid gland during artificial perfusion with control solution or solutions containing either 1 0 mM dibutyryl-cAMP or 1 0 mM theophylline

	Control (mV)	Dibutyryl-cAMP (mV)	Theophylline (mV)
All units	− 6 9±0 6 (180)[a]	−‐8 5±1 1 (90)[a]	−29 9±1 4 (5)[a]
Units below −40 mV	−25 1±0 5 (166)	−25 6±0 8 (79)	−‐6 7±0 9 (45)

[a] Results are presented as mean values±S E. The figures within parentheses indicate the number of measurements

Electrophysiological measurements performed during sustained low rate salivary secretion due to either db-cAMP or theophylline revealed membrane potential values indistinguishable from control values in the same preparation (Table I) Similarly ACh evoked secretory potentials accompanying db-cAMP or theophylline potentiated secretory responses did not differ significantly from secretory potentials under control conditions (Fig 1)

The effect of parasympathetic denervation and atropine on salivary secretion induced or potentiated by dibutyryl cyclic AMP or theophylline

To determine whether induction and potentiation of salivary secretion by db-cAMP or theophylline originated in an action of these agents on the parasympathetic nerve terminals parasympathetic denervation was performed in one animal by section of the auriculotemporal nerve It was found that on the ninth day of denervation both theophylline and db-cAMP were able to induce a sustained low rate salivary secretion Theophylline (db-cAMP was not tested) also produced a potentiation of ACh evoked secretory responses (Fig 3 B) Both potentiation and resting flow in the presence of the drugs were of the same order as under control conditions Similarly resting and secretory potentials were comparable to those encountered in innervated preparations

It appeared therefore that induction and potentiation of salivary secretion by either db-cAMP or theophylline were not mediated by release of transmitter from the parasympathetic nerve endings On the other hand salivation might have been induced or potentiated by the drugs acting directly on the cholinergic receptors This possibility was investigated by studying the effect of a receptor blockade by atropine on the secretion inducing action of for instance theophylline

As a result of this investigation it was found that in 10^{-8} M or lower concentration atropine seemed to have no influence on either ACh induced secretion itself or its potentiation by theophylline (Fig 4) In 10^{-7} M concentration on the other hand the drug was able to completely suppress every response to previously supramaximal doses of ACh In contrast to this 10 M atropine did not to any appreciable extent interfere with the induction of salivary secretion by theophylline This observation was made in experiments in which after pretreatment with 1 0 mM theophylline perfusion with 10 M atropine (in the continued presence of 1 0 mM theophylline) was instituted immediately after a secretory response had been elicited by injection of a supramaximal dose of ACh Under such circum-

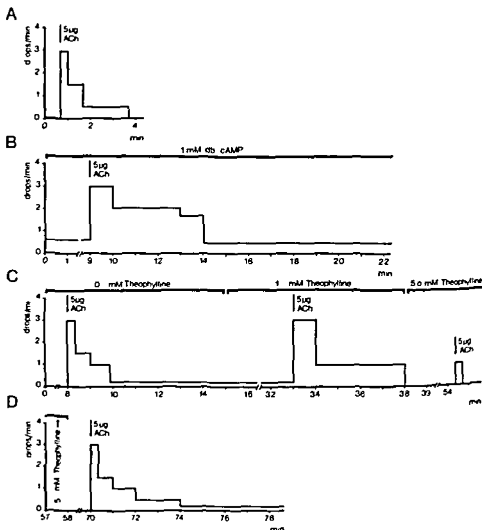


Fig Sali ary secretion in an isolated artif cially perfused parotid gland A secretory response e oked by 5 / g ACh (time of injection marked by arrow) under control conditions B secretory response induced by 1 0 mM db-cAMP and by 5 / g ACh in the continued presence of 1 0 mM db-cAMP C secretory responses induced by arious concentrations of theophyll ne (as indicated by horizontal brackets) and by 5 / g ACh in the presence of various theophylline concentrat ons. D secretory response evoked by 5 / g ACh administered 1 min after perfusion with 5 0 mM theophylline

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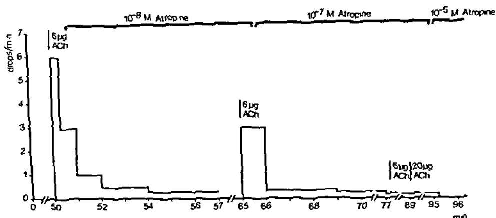


Fig. 4 Salivary secretion in an isolated artificially perfused parotid gland evoked by various amounts of ACh (times of injection marked by arrows) in the permanent presence (from zero time) of 1 0 mM theophylline and in the presence of various concentrations (as indicated by horizontal brackets) of atropine

perfusion with 10^{-7} M atropine was instituted shortly after eliciting a secretory response by injection of a supramaximal dose of ACh It was noted that the initial response was followed by a prolonged phase of slow salivation A simultaneous blockade of both β adrenergic and cholinergic receptors therefore seemed to have no effects on induction of salivary secretion by theophylline

Discussion

The two principal findings yielded by the present investigation are 1) that in the isolated parotid gland of cat intraarterially applied db-cAMP is able both to elicit a slow but often maintained salivary flow and to potentiate ACh-evoked secretory responses and 2) that the same effects can be produced also by intraarterial application of theophylline Keeping in mind that these effects are independent of any receptor blockade and consequently indicative of an intracellular site of drug action it would appear that with the above findings it has been possible to satisfy two of Sutherland s *et al* (1968) criteria for involvement of cAMP as a secondary messenger in hormonal control of cellular activity For this reason it will be presumed in the further discussion that an adenylate cyclase system where cAMP functions as mediator of hormone or transmitter action exists in the parotid gland of cat even though the presence of adenylate cyclase and its proper response to hormonal stimulation has yet to be demonstrated

It is appreciated that the validity of this presumption depends entirely on the specificity of the observed drug actions Special attention has therefore to be paid to the findings that higher concentrations of theophylline inhibit salivary secretion and that the secretory effects of db-cAMP cannot be reproduced by the parent substance cAMP A reversal of the effect of theophylline with increasing concentration is known in a number of biological systems where the function of cAMP as secondary messenger is well established (Rosen and Rosen 1969 Ripoche and Bourguet 1969 Knodell *et al* 1970 Williams and Wolff 1971 Case and Scratcherd 1972) It is implied therefore that in lower concentrations the substance

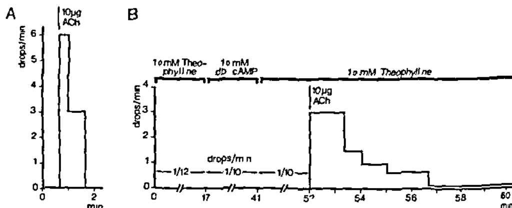


Fig 3 Salivary secretion in a 9 day denervated isolated artificially perfused parotid gland A secretory response evoked by 10 µg ACh (time of injection marked by arrow) under control conditions B secretory responses induced by 1 0 mM theophylline and 1 0 mM db-cAMP (as indicated by horizontal brackets) and 10 µg ACh in the presence of 1 0 mM theophylline

stances the initial response was followed by a prolonged flow of saliva despite a substantial receptor blockade as manifested by the irresponsiveness of the preparation to further ACh injections During this phase of prolonged secretion electrophysiological measurements revealed membrane potentials of normal size as well as complete absence of secretory potentials in response to ACh injections

In 10^{-4} M concentration atropine appeared to exert an inhibitory action even on theophylline induced secretion This was inferred from two experiments where after pre treatment with 10^{-3} M atropine 1 0 mM theophylline was unable to induce any secretory response Additionally it was observed in several cases that theophylline induced secretion was stopped after application of 10^{-4} M atropine (Fig 4)

The effect of a β adrenergic blocker on theophylline induced potentiation of salivary secretion

As there are reports in literature indicating that activation of the cyclic AMP system could be mediated through the β adrenergic receptor (Batzri and Selinger 1973 Robinson Butcher and Sutherland 1968 Scratcherd and Case 1973) experiments were performed to study the effect of a β blockade by INPEA on the secretion inducing action of theophylline

The experiments showed that in concentrations of up to 10^{-4} M INPEA had no influence on either ACh induced secretion itself or on its potentiation by theophylline (Fig 5 A) Only when after pre treatment with 1 0 mM theophylline INPEA was given in 10^{-3} M concentration (in the continued presence of 1 0 mM theophylline) it seemed to be able to slightly diminish the magnitude of ACh evoked secretory responses (Fig 5 A and 5 B) It will be appreciated however that a 10^{-4} M concentration is far above that needed to produce an effective β-adrenergic blockade in the living animal (Emmelin and Holmberg 1967)

In one experiment after pre treatment with 1 0 mM theophylline and 10^{-4} M INPEA

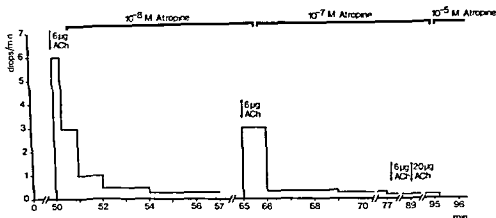


Fig. 4 Salivary secretion in an isolated artificially perfused parotid gland evoked by various amounts of ACh (times of injection marked by arrows) in the permanent presence (from zero time) of 1 0 mM theophylline and in the presence of various concentrations (as indicated by horizontal brackets) of atropine

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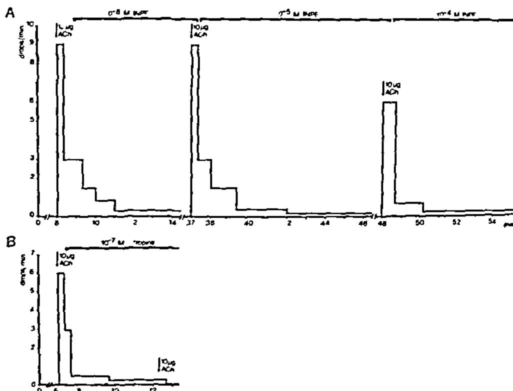


Fig. 5 Salivary secretion in an isolated artificially perfused parotid gland A secretory responses evoked by 10 µg ACh (times of injection marked by arrows) in the permanent presence (from zero time) of 1 0 mM theophylline and in the presence of various concentrations (as indicated by horizontal brackets) of the β-blocker INPEA B secretory response evoked by 10 µg ACh in the continued presence of 1 0 mM theophylline but following exposure to 10^{-4} M INPEA and in the presence (as indicated by horizontal brackets) of 10^{-7} M atropine

does exert its normal phosphodiesterase inhibiting action This appears to be the case in the parotid gland of cat also especially since the typical theophylline effects appear at 5–50 times lower concentrations than the atypical ones The reason why in high concentrations theophylline should inhibit salivary secretion in the cat parotid gland is not evident. The drug might of course lower the activity of adenylate cyclase as in the erythrocyte (cf Sheppard 1970) On the other hand because of its phosphodiesterase inhibiting action it might also give rise to a marked increase in intracellular cAMP concentration which in turn and like in smooth muscle (Andersson and Nilsson 1972, Takagi Takayanagi and Tomiyama 1971) may lead to an effective trapping of intracellular calcium this could underlie the small size of ACh evoked secretory responses in the presence of high concentrations of theophylline provided that calcium acted as a mediator of cholinergic excitation in salivary glands as has been suggested by Petersen (1972)

The inability of cAMP to reproduce the effects of its derivative db-cAMP may seem disturbing in view of previous evidence showing that the two substances may differ in their respective pharmacological actions (Robinson *et al* 1965 Kim Shulman and Levine 1968 Hilz and Tarnowski 1970 Solomon Brush and Kitabchi 1970) However from the

similarity exhibited by the secretory effects of theophylline and db-cAMP respectively it is still likely that in the present preparation the latter substance is acting in the same way as endogenous cAMP The reason why exogenous cAMP appears ineffective could depend then on its well known difficulties to penetrate biological membranes and on its susceptibility to enzymatic hydrolysis (cf Posternak Sutherland and Henison 1962)

The presumption of an adenylate cyclase system in the cat parotid gland naturally raises the question of its localization and functional significance The question of localization is answered partially by the finding that the secretory effects of db-cAMP or theophylline remain unchanged both after parasympathetic denervation and after blocking of all membrane receptors This must mean that the drugs cannot act exclusively in the nerve endings but rather and to a significant extent in the epithelial cells of the gland Recently it has been demonstrated that intraluminally applied cAMP produces a decrease in Na^+ permeability at the luminal face of the ductal epithelium (Young 1973) Even though an action of this kind cannot be excluded in the present preparation it is still difficult to see how it could give rise to a maintained salivary flow as described above It has to be concluded therefore that primarily the secretory effects of db-cAMP and theophylline arise because an adenylate cyclase system is present in the acinar cells of the gland

Regarding the functional significance of such a system it seems reasonable to assume that this lies in participation in nervous control of secretion The assumption naturally leads to the question as to the kind of nervous control sympathetic or parasympathetic in which the adenylate cyclase system might participate No definitive answer can be given to this question at the present stage However in choosing between the two alternatives an involvement of an adenylate cyclase system in parasympathetic control of the gland appears quite unlikely in view of the obvious differences between secretory responses to ACh and to db-cAMP or theophylline respectively

On the other hand several remarkable similarities do exist between the secretory effects of db-cAMP or theophylline and those of sympathetic nerve trunk stimulation (cf Ekstrom and Emmelin 1974) In both cases secretion is slow it appears after some latency and has a tendency to decline in spite of continued stimulation or drug exposure respectively Also in both cases secretion takes place in the absence of any measurable acinar membrane potential changes (cf Fritz and Bothelho 1969 b) Finally there is a strong resemblance between the potentiating influence of db-cAMP or theophylline on ACh evoked secretion and the recently noticed potentiating effect of cholinergic activation on secretion elicited by sympathetic nerve trunk stimulation (Emmelin personal communication)

Under these circumstances it is conceivable that the functional role of an adenylate cyclase system consists in mediating sympathetic excitation The implication of this would be that the adenylate cyclase system was coupled to the β adrenergic receptor of the membrane In this respect the cat parotid gland would resemble the parotid gland of the rat where an adenylate cyclase system controlled by the β adrenergic receptor seems to be involved in the regulation of amylase secretion (Bdolah Ben Zvi and Schramm 1964 Bdolah and Schramm 1965 Schramm and Naim 1970)

From the discussion above it follows that the parotid gland of cat was in possession of two excitation mediating mechanisms an adenylate cyclase system for sympathetic and a

different possible calcium operated system for parasympathetic excitation This type of functional organisation would be shared also by for instance the pancreas of cat where excitation by secretin is mediated by an adenylate cyclase system whereas cholinergic excitation or excitation by cholecystokinin pancreozymin appears to be mediated via a different mechanism (Case and Scratcherd 1972 a and b) The presence of two secretion regulating systems must not, however imply functional independence between these systems. They could be linked to each other by a coupling factor which may be thought of as being calcium in the case of the cat parotid gland Calcium has been suggested as mediator of cholinergic excitation in the cat salivary gland (Petersen 1972) and is also known to possess adenylate cyclase activating properties (Rasmussen 1970) by which it could modify mediation of adrenergic excitation It is possible that the existence of a coupling factor of the discussed kind underlies the mutually potentiating effects of the two secretion controlling systems which have been described above and elsewhere (Emmelin to be published) and which may be of importance in achieving the correct proportions of salivary constituents in the living animal

This study was supported by grants from the Swedish Medical Research Council (project No 539) The experiments were conducted during the term of M E Fritz as a Senior Exchange Fellow of the Swedish Medical Research Council (project No B74-60R4355) The authors wish to thank Professor N Emmelin for valuable advice and critique and for performing the denervation described above The able assistance of Miss K Borglid and Mr L Sjolin is gratefully acknowledged

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Acta physiol scand 1975 93 364–377

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Adenosine Triphosphate Dependent Calcium Uptake by Subcellular Fractions from Bovine Neurohypophyses

By

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Received 27 august 1974

Abstract

RUSSELL J T and N A THORN *Adenosine triphosphate dependent calcium uptake by subcellular fractions from bovine neurohypophyses* Acta physiol scand 1975 93 364–377

Bovine neurohypophyses were fractionated by differential and density gradient ultracentrifugation and the Ca^{2+} uptake and ATPase activities in the microsomal mitochondrial and secretory granule fractions were studied. The microsomal and mitochondrial fractions accumulated Ca^{2+} in the presence of ATP. The accumulation by the latter per mg protein was at least twice as large as by the former. This Ca^{2+} accumulation was accompanied by liberation of inorganic phosphate (P_i). In the presence of sodium azide (2 mM) Ca^{2+} uptake and P_i liberation were inhibited in the mitochondrial but not in the microsomal fraction. Further studies of the microsomal fraction revealed that the ATP-dependent Ca^{2+} uptake and P_i liberation activities were temperature and pH-dependent and required Mg^{2+}. Both activities were stimulated by very low concentrations of Ca^{2+} (1–10 μM) and were inhibited by EGTA (mM). N-ethylmaleimide (mM) inhibited both the Ca^{2+} uptake and ATPase activities of the microsomal fraction. These results suggest the presence of a membrane ATPase that is stimulated by both Ca^{2+} and Mg^{2+}. It is suggested that the observed Ca^{2+} uptake activities are involved in maintaining a low axoplasmic free Ca^{2+} concentration thus playing an important role in the release mechanism of vasopressin by the neurosecretory terminals

The release of vasopressin from the neurohypophysis seems to be the best studied *terminal* function of any neurosecretory system. Calcium ions play an important role in stimulus-secretion coupling for this vasopressin release (Douglas and Poisner 1964 a b Russell and Thorn 1974 a b). Entry of Ca^{2+} ions across the terminal membrane has been shown to be essential for stimulated secretion of vasopressin and oxytocin from isolated neurohypophyses (Russell and Thorn 1972 1974 b Dreifuss *et al* 1973). Further treatment of isolated rat neurohypophyses with the SH reagent N-ethylmaleimide (Douglas Ishida and Poisner 1965) which seems to release intracellularly bound Ca^{2+} (Russell Warberg and Thorn 1974) or with the ionophore A 23187 (Russell Hansen and Thorn 1974) which increase trans membrane Ca^{2+} transport can induce secretion of vasopressin. This suggests that in the absence of applied cell membrane depolarization increasing the intracellular free Ca^{2+} concentration alone can trigger secretion

1 With the technical assistance of B L CHRISTENSEN

The extracellular Ca^{2+} concentration exceeds the estimated intracellular concentration of free Ca^{2+} by a factor of 10^4 or more (Thorn 1974) and the intracellular free Ca^{2+} concentration has to be maintained below a critical level in resting neurosecretory cells for such a system to work

In ordinary nerve cells regulation of intracellular Ca^{2+} concentration may be achieved by an active extrusion mechanism (Baker and Glitsch 1973) and/or by a Na^{2+}–Ca^{2+} exchange over the axoplasmic membrane (Baker *et al* 1969) Like in muscle cells the accumulation of Ca^{2+} by mitochondria and endoplasmic reticulum in nervous tissue have been thought to contribute to the removal of Ca^{2+} from the cytosol (Otsuka Ohtsuki and Ebashi 1965 Lieberman Palmer and Collins 1967 Robinson and Lust 1967 Lehninger 1970 a DeMeiss Rubin Atschul and Machado 1970)

Direct evidence for an ATP dependent Ca^{2+} accumulation in membrane fractions (microsomes) or other fractions isolated from neurohypophyseal tissue has been lacking

Methods and Materials

Pituitary glands were obtained at the Copenhagen Public Slaughterhouse from cows within 5 to 15 min of death and transported in approximately 30 min to the laboratory in an ice bucket The neural lobes were then isolated and washed in the homogenizing medium (0 25 M sucrose with 0 02 M N tris (Hydroxy methyl)methyl 2 amino-ethane sulfonic acid (TES)) at pH 7 0 In some later experiments the superficial 1 mm of tissue from the anterior and ventral surfaces of the neural lobes were removed to eliminate any contamination of the preparation with pars intermedia cells 6 whole neural lobes with a total wet weight of approximately 2 5 g were used for each fractionation

Subcellular fractionation

Homogenization The isolated neural lobes were minced in ice-cold homogenizing medium using a pair of scissors The minced tissue was homogenized in a Potter Elvehjem type homogenizer (radial clearance 0 013 to 0 018 mm) by 6 upward and downward strokes at 900 to 1 000 RPM The homogenizer was surrounded by ice and all further operations were carried out at 0–5 C. The homogenate was made up to a 10 suspension in homogenizing medium for differential centrifugation

Differential centrifugation For obtaining the different subcellular organelles in a fairly pure form the method described by Dean and Hope (1967) was used with slight modifications The homogenate was taken to a speed of 3 000 g in a MSE 18 High Speed centrifuge at 4 C as fast as possible and allowed to run for 30 s and then was allowed to decelerate without braking. The sediment was termed fraction 1 The supernatant was centrifuged at $4\,000 \times g$ for 15 min yielding fraction 2 Fraction 3 was obtained from the second supernatant by centrifuging at 6 000 g for 15 min in a Beckman L2–65K ultracentrifuge at 4 C The supernatant was spun at 105 000 g for 120 min The sediment was termed fraction 4 The final supernatant was termed fraction 5 The sediments were suspended in 2 ml of the reaction medium (*vide infra*)

Density gradient centrifugation The purified secretory granule fraction was obtained from fraction 3 by density gradient centrifugation according to the method of Dean and Hope (1967) 5 fractions were obtained they were termed A to E from the top of the tube (Fig 1)

Assay of markers for subcellular organelles

For the analyses of markers the differential centrifugation sediments (1 to 4) were suspended in 0 ml of redestilled water whereas the other fractions were used undiluted All fractions were sonicated at 60 W for 10 s repeated 3 times with an interval of 0 s on a Sonifer® Model B 12 (Branson Sonic Power Co Connecticut) In cases where bioassays of vasopressin or oxytocin were done the sonicated fractions were extracted by boiling for 3 min in 0 5 M acetic acid in 0 9 NaCl solution

Bioassay of vasopressin Vasopressin was assayed by the rat blood pressure method of Dekanski (195) with the modifications described by Russell and Thorn (1974 a)

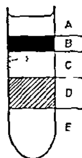


Fig 1 Appearance of bands after density gradient centrifugation of fraction 3 which was obtained by differential centrifugation A 1 ml sample of fraction 3 suspended in the homogenizing medium (0 75 M sucrose in 0 mM TES buffer) was layered on a discontinuous density gradient (from 1 30 to 2 00 M sucrose) Centrifugation was at 101 000 × g for 60 min The lines indicate the position at which the tube was cut to obtain the 5 fractions A–E.

Bioassay of oxytocin Oxytocic activity was assayed by following the isometric method of Ryd n and Sjoholm (1968) using isolated rat mammary strips The strips were kept in an organ bath in Tyrode solution of the composition described by Perry (1968) under an initial tension of approximately 700 mg The tension developed was monitored by a TLK 5/0 01 (Torsten Ljungstrom AB Ringvägen Sollentuna Sweden) strain gauge and recorded on a Servogor (RE 511) recorder The strain gauge gave a linear response o er the range needed for the assay (00 to 300 mg)

Succinate dehyd ogenase (EC 1 3 99 1) was determined as described by Pennington (1961) using the iodo nitro tetrazolium (INT) reductase reaction with the modifications suggested by Porteous and Clark (1965).

Fumarase (1 maleate hydro-lyase EC 4 2 1 2) was estimated by the method of Racker (1950) with the following modifications The reaction mixture contained 125 μl enzyme and 125 μl KH PO NaOH buffer containing 0 05 M maleate at pH 7 4 The Δ E at 240 nm 3 min after the addition of the enzyme was read against a blank (quartz microcuvettes) containing no maleate The readings were then converted to μM fumarate liberated using a standard curve The standard fumarate concentrations were made in KH_4PO_4 NaOH buffer containing maleate and read against buffer without maleate One unit of fumarase was defined as the activity that formed 1 μM fumarate in 3 min under the conditions of the assay

NADPH Cytochrome C reductase (EC 1 6 2.3) was determined as described by Sottocássa et al (1967) with 0 15 mM rotenone as suggested by DeJong and Hülsmann (1970)

Glucose-6-Phosphatase (EC 3 1 3 9) was determined according to Nordlie and Arion (1966) Inorganic phosphate l berated was assayed by the method of Fiske and Subbarow (19_5)

β-Glucuro idase (EC 3 1.31) was estimated by the method of Talalay Fishman and Huggins (1946).

Lactate dehydrogenase (EC 1 1 1 27) was estimated according to Wroblewski and LaDue (1955) The reaction mixture contained 0 1 ml enzyme 1 0 ml KH_2PO_4–NaOH buffer containing 0 31 mM pyru ate pH 7 4) and 0 0 5 ml NADH (9 mM)

Protein was estimated by the method of Lowry et al (1951) with the mod fications of Lous Plum and chou (1956)

Measurement of Ca^{2+} uptake activity

The reaction medium for the measurement of Ca^{2+} uptake had the following composition (mM) KCl 124 0 NaCl 5 0 $MgCl_2$ 5 0 TES 0 0 (pH 7 0) and 5 10^{-6} M $CaCl_2$ of which 9 1 10^{-} M was $^{45}CaCl_2$. The volume of the total reaction mixture was usually 20 ml unless otherwise stated The reaction was carried out at room temperature (21 to 4 C) with continuous stirring The reaction was started by the addition of the suspension containing the particulate protein The final protein concentrations in the reaction mixtures are indicated in the legends of the figures. To stop the reaction 2 0 ml of the mixture was applied on a mill pore filtration apparatus (XX100 2530 Millipore Corporation Bedford Mass) using type HA filters of 0 45 μm pore size (Martonosi and Feretos 1964) The filtration was complete in to 3 s and no measurable particulate protein escaped into the f ltrates The filters were washed with _0 ml of a solut on of a similar composition to that of the reaction mixture with no $^{45}Ca^{2+}$ and were then placed in a polyethylene counting vials (Scintec Gentofte Denmark) To determine the specific activity of $^{45}Ca^{2+}$ in the reaction mixture 0 1 ml aliquots were placed on filters in vials and counted The Ca^{2+} concentrations of the media were estimated flourimetrically (on Hattingberg et al 1968) Triplicate samples of a complete reaction mixture without the particulate protein were filtered to estimate the $^{45}Ca^{2+}$ counts adsorbed to the filter discs, which was subtracted from the counts in the presence of particulate protein Ca^{2+} counts taken up by the particulate protein was converted to nM Ca taken up per mg protein u ing the specific act ity of $^{45}Ca^{2+}$ in the reaction mixture

$^{45}Ca^{2+}$ *counting* 2 ml of counting solution (PPO 5 0 g/l POPOP 0 067 g/l Triton X 100 Toluene 1 2 v/v was added to the counting vials The samples were counted in a Packard Tri–Carb liquid scintillation spectrometer (model 3375) Details of the method and its efficiency and reliability have been published previously (Russell and Thorn 1974 a)

Measurement of ATPase activity The liberation of inorganic phosphate to the reaction mixture by the presence of the particulate protein was taken as indication of ATP hydrolysis 1 ml aliquots of the filtrates were transferred to stoppered glass tubes kept in a dry ice acetone mixture (−87 C) and stored for inorganic phosphate (P_i) analysis P_i determination was made according to the method of Parvin and Smith (1969) The results were expressed as nM P_i liberated per mg protein at the given time intervals.

Technique for studying subcellular distribution of $^{45}Ca^{2+}$ in slices of bovine neurohypophyses

Slices from bovine neural lobes were prepared and transported to the laboratory as described previously (Russell and Thorn 1974 a) The equilibrated slices were incubated at 37 C in a Tris buffered control or high (56 mM) $(K)_o$ medium containing $^{45}Ca^{2+}$ (10 μCi/ml 9–11 μCi μM $CaCl_2$) for 30 min (Russell and Thorn 1974 a) At the end of 30 min they were blotted minced and homogenized in ice cold 0 3 M sucrose solution Subcellular fractions were obtained by the differential centrifugation procedure outlined above using a nonbuffered 0 3 M sucrose solution Aliquots of the fractions were placed in quartz boats and ashed at 700 C for 3 min in the biological material oxidizer (Beckman Instruments Inc Fullerton) The ash was dissolved in 2 ml of 0 1 N HCl and aliquots were counted as described previously (Russell and Thorn 1974 a) In another set of experiments the slices incubated in $^{45}Ca^{2+}$ were washed for 60 min in a La (12 mM) containing medium as described earlier (Russell and Thorn 1974 a) and subjected to homogenization and differential centrifugation

Chemicals All chemicals were analytical grade Adenosine 5 triphosphate (ATP) Cytochrome-C Iodo nitro tetrazolium (INT) Nicotinamide adenine dinucleotide phosphate reduced form (NADPH) N ethylmaleimide (NEM) N tris (hydroxymethyl) methyl 2 aminoethane sulfonic acid (TES) 3-(N morpholino)propane sulfonic acid (MOPS) and phenolphthalein β glucuronidate were obtained from Sigma Chemical Co St Louis Mo USA

Phosphoneolpyruvate and pyruvate kinase were obtained from Boehringer Mannheim Germany

Ethylene bis-glycol tetra acetic acid (EGTA) and Fiske Subbarow reducing reagent were purchased from E. Merck Germany 1 4-bis 2(methyl 5 phenyloxazolyl)-benzene (POPOP) and 2 5 diphenyloxazole (PPO) were obtained from Packard Instrument Co

$^{45}Ca^{2+}$ as $CaCl_2$ in aqueous solution was obtained from Radiochemical Centre Amersham (Batch Nos CES3 12A CES3 9D6)

Results

Purity of the subcellular fractions

The distributions of vasopressin oxytocin and various enzyme activities in the fractions obtained by differential and density gradient centrifugations are presented in Fig 2 The highest specific activities of vasopressin and oxytocin were found in fraction D suggesting a high concentration of secretory granules As described by Dean and Hope (1967) mitochondria were largely found in fraction 2 and fraction B as shown by the distribution of succinate dehydrogenase and fumarase Ca^{++} uptake by mitochondria was studied using fraction 2 except on one occasion where fraction B was used This gave comparable results Fraction 4 was found to contain the highest specific activities for the two microsomal markers NADPH Cytochrome C reductase and glucose 6-phosphatase The contamination of the microsomal fraction with mitochondria was less than 1% as shown by the marker enzyme distribution pattern However the microsomal fraction was found to contain significant amounts of β glucuronidase activity indicating a possible contamination with lysosomes or disrupted lysosomal membranes The second possibility is more likely as β-glucuronidase has been shown to be largely associated with lysosomal membranes (Gianetto and DeDuve 1955)

Fig *The distribution of vasopressin oxytocin and marker enzymes in subcellular fractions from bovine neurohypophyses obtained by differential and density gradient centrifugation*

RSA (relative specific activity) = recovered activity / recovered protein

Each bar represents mean S E The number of experiments are indicated in parentheses

Ca uptake and ATPase activities of microsomes mitochondria and secretory granules

In all experiments the Ca uptake was measured simultaneously in two reaction vessels one with and the other without ATP The binding of Ca to the particulate protein in the ATP free medium was small (approximately 1 0 nmol/mg protein) and was subtracted from the accumulation in the presence of ATP A similar correction was applied for the P_i libera tion data The ATP dependent calcium uptake increased up to 30 min and approached a

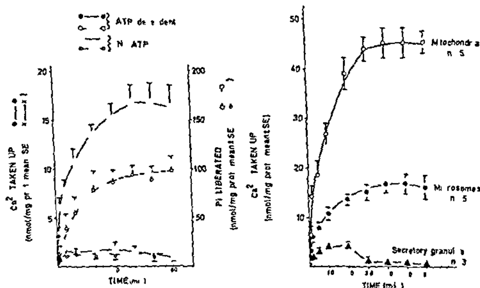


Fig 3 Ca^{2+} uptake and P_i liberation by microsomes from bovine neurohypophyses The microsomes were incubated at 22 C in the standard reaction medium as described in Methods and Materials with or without ATP (2 mM) PEP and PK were not present Protein concentration in the reaction mixtures were 115 to 160 μg/ml ATP-dependent values represent activity in the presence/absence of ATP Values are mean ± S E n = 5

Fig 4 ATP-dependent Ca^{2+} uptake by neurohypophyseal mitochondria (fraction 2) microsomes (fraction 4) and secretory granules (fraction D) The incubation was carried out in the standard reaction medium at 22 C in the presence of ATP (2 mM) PEP (2 mM) and PK (10 μg/ml) The protein concentrations of the reaction mixture were *Mitochondria 151 to 2.5 μg/ml microsomes 115 to 160* μg/ml and secretory granules 103 to 189 μg/ml

steady state thereafter (Fig 3) In a number of instances a loss of accumulated $^{45}Ca^{++}$ from the microsomes was noticed (Fig 11) after 40 min incubation The total amount of Ca^{++} taken up was not different in reaction mixtures with ATP (2 mM) alone or with ATP and regenerating system (phosphoenol pyruvate (PEP) and pyruvate kinase (PK)) However in all experiments phosphoenol pyruvate (2 mM) and pyruvate kinase (10 μg/ml) were present unless otherwise indicated The microsomal ATP-dependent Ca^{++} uptake activity was not enhanced by the presence of 5 mM oxalate in the reaction mixture (4 expts)

The ATP-dependent Ca^{++} accumulation in mitochondria was markedly higher than that found in the microsomes (Fig 4) In both the mitochondrial fraction and the microsomal fraction the increase in P_i liberation roughly parallelled the Ca^{++} accumulation (Fig 3 11)

The secretory granule fraction showed negligible and not maintained Ca^{++} uptake activity (Fig 4) and there was no measurable P_i liberation

Characterization of microsomal fraction Ca^{++} uptake and ATPase activities

Effect of temperature The microsomal fraction Ca^{++} uptake and ATPase activities were found to be temperature dependent (Fig 5) The total amount of Ca^{++} accumulated near steady state at 5 0 22 0 and 37 0 C were 5 8 16 2 and 33 5 nmol/mg protein respectively The P_i liberation presented a similar picture

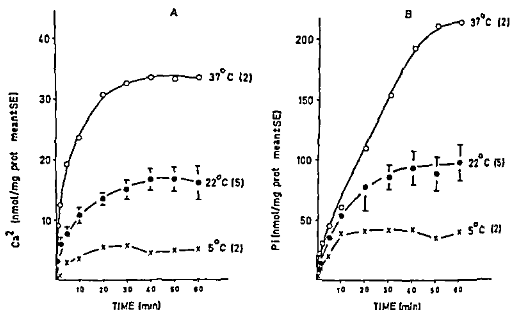


Fig 5 Effect of temperature on mean ATP dependent Ca^{2+} uptake (A) and P_i liberation (B) by neurohypophyseal microsomes The incubation conditions were the same as described in Fig 4 The protein concentration of the reaction mixtures were 155 to 165 μg/ml Numbers in parentheses indicate number of experiments

Effect of pH The microsomal Ca^{2+} uptake and ATPase activities were measured over a pH range from 5 to 8 5 using MOPS buffer (3 (N morpholino) propane sulfonic acid pK 7 2) Glycine–NaOH buffer was used for pH between 9 to 10 With both these buffers the steady state uptake (30 min) at pH 7 0 was identical with that found in the TES buffered medium The results are shown in Fig 6 Ca^{2} uptake activity had a sharp peak at pH 7 0 No uptake was seen at pH 10 0 However the ATPase activity (not illustrated in Fig 6) showed irregular pH dependency probably because of the presence of other phosphatases in the preparation

Dependence on Ca^{2} The amount of Ca^{2} accumulated in 30 min (22 C) increased with increasing medium Ca^{2} concentration up to 0 075 mM and tended to level off thereafter (Fig 7) The figure also shows that the Ca^{2} uptake was linearly related to the log Ca^{2} concentration of the medium The accompanying ATPase activity however showed a sharp rise between 1 and 5 μM Ca^{2} concentrations and thereafter did not increase with increasing medium Ca^{2} concentrations

In 2 expts the microsomal fraction was incubated in a Ca^{2} free medium containing only tracer Ca^{2} (^{45}Ca) for 10 min at which time Ca^{2} was added to a final concentration of 0 05 mM As shown in Fig 8 the Ca^{2} uptake and ATPase activities were markedly low in the absence of Ca^{2} On addition of Ca^{2} both activities showed a sharp increase The rapid apparent loss of accumulated Ca^{2} calculated here may reflect tracer equilibration due to the lowering of $^{45}Ca^{2}$ specific activity in the medium on addition of ^{4} Ca^{2}

Ca^{2} uptake and ATPase activities of the microsomal fraction were negligible in a medium

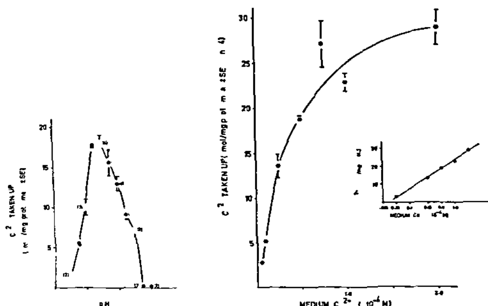


Fig 6 The effect of pH on ATP-dependent Ca^{2+} uptake by neurohypophyseal microsomal fraction The microsomes were incubated at 2. C for 30 min in standard Ca^{2+} uptake medium prepared with MOPS buffer for pH between 5 0 to 8.5 Glycine–NaOH buffer was used for pH 9 0 to 10 0 The volume of the reaction mixtures was 2 5 ml The protein concentrations of the reaction mixtures were 150 to .50 μg/ml The values are mean or mean ± S E. Figures in parentheses indicate the number of experiments.

Fig 7 The effect of medium Ca^{2+} concentration on ATP-dependent Ca^{2+} uptake by neurohypophyseal microsomes The reaction was carried out at .2 C in the standard Ca^{2+} uptake medium The volume of the reaction mixtures was 2 5 ml The inset shows the values plotted on a semilogarithmic scale The protein concentrations of the reaction mixtures were 170 to 00 μg ml The values are mean of 4 independent observations ± S E.

containing EGTA (2 mM Fig 9) The P_i liberation in the presence of EGTA was unmeasurable

Dependence on Mg^{2+} The Ca^{2+} accumulation and ATPase activities showed absolute dependence on the presence of Mg^{2+} in the medium In a Mg^{2+} free medium both the activities were absent (Fig 10)

Effect of N-ethylmaleimide The sulfhydryl reagent N-ethylmaleimide (0 1 – 2 mM) dose dependently inhibited the Ca^{2+} uptake and ATPase activities of the microsomal fraction The Ca^{2+} accumulation showed an initial rise up to 10 min of incubation and sharply fell to very low levels thereafter (Fig 9) The P_i liberated at steady state (40 min) in the presence of NEM was 16 71 ± 6 04 nmol/mg protein as against 159 73 ± 30 66 nmol/mg protein in the control experiments (Mean ± S E n = 3 and 5 respectively)

Effect of azide The presence of sodium azide (2 mM) in the incubation medium did not have any effect on the microsomal Ca^{2+} accumulation or ATPase activities However the same concentration of azide inhibited the mitochondrial Ca^{2+} accumulation and P_i liberation (Fig 11)

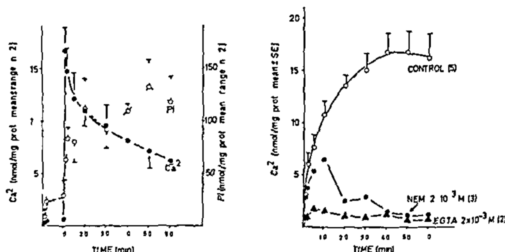


Fig 8 The effect in 2 expts of *adding* Ca^{2+} *to a* Ca^{2+} free incubation medium on mean ATP-dependent Ca uptake and P_i liberation by neurohypophyseal microsomal fraction The microsomes were incubated at 22 C in the standard reaction medium (initial volume 30 ml) containing only $^{45}Ca^{2+}$ (0 91 μM) for 10 min at which time Ca^{2+} was *added in 20 μl to a final* concentration of 0 05 M The protein concentrations of the reaction mixtures were 117 to 232 μg/ml The vertical bars indicate the ranges

Fig. 9 Effect of EGTA (? mM) and N-ethylmaleimide (NEM) (2 mM) on ATP-dependent Ca^{2+} uptake by neurohypophyseal microsomal fraction The microsomes were incubated at 22 C in the standard reaction medium containing the *drugs* *The final protein* concentrations of the reaction mixtures were 97 to 1?? μg/ml

Subcellular distribution of ^{45}Ca *in slices incubated in a medium with 4 8 or 56 0 mM K*

Fraction 2 containing mostly mitochondria (see marker enzyme distribution Fig 2) was found to contain the highest activity of $^{45}Ca^{2+}$ (Table I) When the $^{45}Ca^{2+}$ distribution in fractions obtained from slices labelled in the control or high $(K)_o$ media was compared)

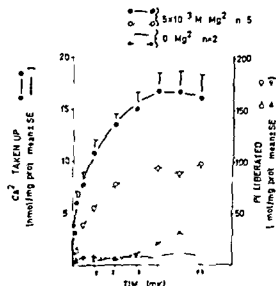


Fig. 10 ATP-dependent Ca^{2+} uptake and P_i liberation in the presence and absence of Mg^{2+} Incubation was carried out at C in standard Ca^{2+} uptake medium containing 5 mM or no Mg^{2+} The protein concentrations of the reaction mixtures were 15 to 173 μg ml

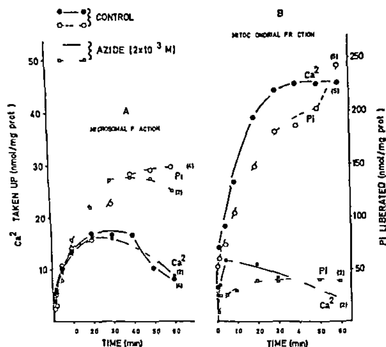


Fig 11 Effect of sodium azide (2 mM) on ATP-dependent Ca^{2+} uptake (closed symbols) and P_i liberation (open symbols) by neurohypophyseal microsomal (A) and mitochondrial (B) fractions The incubation was carried out at 22 C in standard medium The final protein concentrations in the reaction mixtures were for microsomes 139 to 203 μg/ml for mitochondria, 154 to 185 μg/ml The numbers in parentheses indicate number of experiments

there was no significant difference when the slices were not washed in La^{3} containing medium prior to fractionation In the washed slices however the microsomal fraction (4) of high (K) labelled slices showed a significant increase ($p < 0.05$) in tracer concentration

Discussion

The results presented here demonstrate that neurohypophyseal membrane structures *accumulate calcium by an ATP-dependent process* The distribution of fumarase and succinate dehydrogenase (mitochondrial markers) and glucose 6-phosphatase and NADPH cytochrome C reductase (microsomal markers) show that the membrane structures in the microsomal fraction are not contaminated with mitochondrial membranes to any significant degree Further the inability of sodium azide to inhibit the microsomal Ca^{2+} uptake (Fig 11) can be taken as evidence that the mitochondrial contribution to the Ca^{2+} uptake activity found in the microsomal fraction is negligible The inability to retain the accumulated Ca by the particulate protein observed in some preparations (Fig 11) after 30 min incubation may have been due to the presence of contaminating lysosomal enzymes (Fig. 2) which may damage membrane vesicles

TABLE I Distribution of $^{45}Ca^{2+}$ in subcellular fractions of brain neurohypophyseal slices labelled in control and high $(K)_o$ (56 mM) medium Slices of bovine neurohypophyses were incubated in ^{45}Ca containing Tris buffered medium at 37 C for 30 min with or without 56 mM K^+ The distribution of tracer in the subcellular fractions were estimated after ashing aliquots in the Biological Material Oxydizer

$$\text{RSA (relative specific activity)} = \frac{\text{recovered activity}}{\text{recovered protein}}$$

The values are mean ± S E. The numbers in parentheses indicate number of experiments. NS = Not significantly different

Fraction	Slices fractionated immediately after labelling (RSA)			Slices fractionated after 60 min wash in $LaCl_3$ (12 mM) following labelling (RSA)		
	A Control (6)	B High $(K)_o$ (7)	p (A vs B)	A Control (4)	B High (K) (4)	p (A vs B)
1	1 18 ± 0 16	1 15 ± 0 10	NS	0 93 ± 0 22	1 05 ± 0 04	NS
2	1 42 ± 0 16	1 67 ± 0 14	NS	1 71 ± 0 25	1 69 ± 0 09	NS
3	0 90 ± 0 17	1 19 ± 0 15	NS	0 81 ± 0 04	0 97 ± 0 05	NS
4	1 39 ± 0 93	1 30 ± 0 44	NS	0 78 ± 0 07	1 26 ± 0 0	< 0 05
5	0 75 ± 0 10	0 60 ± 0 06	NS	0 93 ± 0 15	0 60 ± 0 05	NS

As has been suggested for skeletal and smooth muscle (Lehninger 1970 b Batra 1973) in the neurohypophysis also mitochondria may play a role in reducing intraterminal free Ca^{2+} concentration Ca^{2+} uptake activity of the mitochondria was at least twice as high as observed in the microsomal fraction (Fig 4) This is in agreement with previous observations in other types of nervous tissue (Lust and Robinson 1970 Carafoli and Lehninger 1971 Lazerewicz, Haljamäe and Hamberger 1974) Possibly the differences would be even higher if uptake for mitochondria could be measured per unit outer membrane protein The mitochondrial Ca^{2+} uptake in the present study was smaller than that reported for brain tissue from rats and rabbits in the above mentioned studies

The absence of Ca^{2+} uptake activity in the granule fraction is not due to the presence of a high concentration of sucrose in the reaction mixture derived from the density gradient fraction D Both the microsomal and mitochondrial fractions after suspension in sucrose solution of the same concentration retained the Ca^{2+} uptake activity The neurohypophyseal secretory granules have been shown to have a high concentration of Ca^{2+} (Vilhardt and Thorn unpublished) This Ca^{2+} appears to be in a form which allows little exchange

The ATP-dependent Ca^{2+} uptake in the microsomal fraction was accompanied by liberation of inorganic phosphate Mg^{2+} and Ca^{2+} were required for the optimal level of both activities and the concentrations of calcium that stimulated the accumulation were of a magnitude likely to occur in the axoplasm Further the presence of NEM inhibited both Ca^{2+} uptake and P_i liberation to the same extent The P_i liberation was increased markedly on addition of Ca^{2+} to a Ca^{2+} free incubation medium (Fig 7) These pieces of evidence suggest that a Ca^{2+}-Mg^{2+} activated ATPase activity is associated with the Ca^{2+} uptake However the contribution to the P_i liberation by other phosphatases in the preparation cannot be excluded A Ca^{2+} activated and a Mg^{2+} activated ATPase in the microsomal fraction of bovine neurohypophyses have been reported previously (Vilhardt and Holmer

1972 Vilhardt and Hope 1974) This ATPase has properties resembling those shown by the Ca^{2+} accumulating systems found here NEM inhibited the Ca^{2+} uptake and associated ATPase activities of neurohypophyseal microsomes (Fig 9) Thus in the intact nerve terminal NEM could bring about increased intracellular free-Ca^{2+} concentrations by inhibiting the uptake of Ca^{2+} by the endoplasmic reticulum and trigger secretion as suggested previously (Russell Warberg and Thorn 1974)

The microsomal fraction would be composed of shorn off pieces of the plasma membrane and cytoplasmic membranes (*e g* endoplasmic reticulum) The broken membranes are known invariably to form enclosed semipermeable vesicles (Wallach and Lin 1973) It is not possible to distinguish whether the Ca^{2+} uptake activity observed here is by one or the other component or both The Ca^{2+} uptake activity observed here may be binding of Ca^{2+} by the membrane structures a transport of Ca^{2+} into the vesicular lumen or a combination of both ATP may preferentially increase the binding of Ca^{2+} to the membrane structures or may promote active transport of Ca^{2+} into the vesicular lumen or both

The data presented here demonstrate that the microsomal fraction from bovine neurohypophyses accumulates Ca^{2+} in much the same way as the microsomes derived from skeletal muscle (Martonosi and Feretos 1964) cardiac muscle (Repke and Katz 1972) and uterine muscle (Carsten 1969) A similar activity has been demonstrated in red cell membranes (Cha Shin and Lee 1971) and platelet membranes (Robblee Shepro and Belamarich 1973) Microsomes prepared from brain tissue and peripheral nerves also accumulate Ca^{2+} (Otsuka Ohtsuki and Ebashi 1965 Lieberman Palmer and Collins 1967) Similar observations have been reported in membrane structures from secretory tissues of adrenal medulla (Poisner and Hava 1970) and submandibular glands (Nijjar and Pritchard 1973) Unlike in the sarcotubular membranes the Ca^{2+} uptake activity of neurohypophyseal microsomes was not enhanced by oxalate Similar lack of oxalate effect has been reported in brain microsomal Ca^{2+} uptake (Otsuka Ohtsuki and Ebashi 1965 Yoshida Kadota and Fugisawa 1966 Robinson and Lust 1968)

Previous studies (Russell and Thorn 1974 a) have shown that $^{45}Ca^{2+}$ is taken up from the medium to slices of ox neurohypophyses on stimulation by 56 mM K^+ and there were indications that part of this uptake took place to a subcellular compartment The results of the present experiments on subcellular distribution of $^{45}Ca^{2+}$ in slices appear to support this conclusion

The ATP-dependent Ca^{2+} accumulation by the microsomal and mitochondrial fraction demonstrated in this study may play an essential role in regulating the axoplasmic concentration of free Ca^{2+} which seems to determine the release of the hormones

This work was supported by grants from the Danish Medical Research Council, No disk Insulinfond and the Danish Foreign Ministry (DANIDA)

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Clearance of Some Quaternary Amines from the Spinal Subarachnoid Space*

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Received 2 September 1974

Abstract

AQUILONIUS S M., B WINBLADH and S ZILLÉN *Clearance of some quaternary amines from the spinal subarachnoid space* Acta physiol scand 1975 93 378–384

The spinal subarachnoid space was perfused with artificial cerebrospinal fluid (CSF) from the low lumbar level to the middle thoracic level or to the cisterna magna in anesthetized rabbits ^{3}H-choline ^{3}H methyl atropine or ^{3}H-decamethonium with carrier in different concentrations was added to the perfusate together with ^{14}C inulin the latter serving as a marker of the dilution of the perfusate by original CSF Choline was eliminated from the perfusate partly by a saturable mechanism probably by an uptake into the spinal cord About 15 per cent of the radioactivity of the choline infused was recovered from the spinal cord mainly as phosphorylcholine betaine and phospholipids Amphetamine decreased the elimination of choline from ventriculocisternal perfusates and partly inhibited the uptake of choline in rabbit choroid plexus *in vitro* In contrast amphetamine did not influence the saturable elimination of choline in the perfusion Neither methylatropine nor decamethonium was eliminated from the perfusate by a saturable mechanism in the lumbothoracic perfusions However in perfusions including the cisterna magna methylatropine was partly eliminated by such a mechanism The concentration of radioactivity in fourth ventricular choroid plexa suggested this structure to be responsible for the saturable part of the elimination In conclusion there is no active removal of quaternary amines in general from spinal CSF like the choroid plexus mediated clearance from ventricular CSF

Several types of compounds including some physiologically and pharmacologically important substances are cleared out of the cerebral ventricles into plasma by a carrier mediated transport (For references see Eriksson and Winbladh 1971) The choroid plexa have been claimed to be the main site for this transport (Aquilonius and Winbladh 1972) The transport permits the maintenance of a steady-state concentration of a substance in cerebrospinal fluid (CSF) which is lower than that of plasma In turn a low CSF concentration favours a downhill diffusion of a substance from the brain tissue into CSF Such a mechanism called the sink action of CSF (Davson 1967) has actually been shown for some sub-

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* A preliminary report of the present paper was given at the XVI Meeting of the Scandinavian Pharmacological Societies 197 (Aquilonius Winbladh and Zillén 197)

stances (for review see Lorenzo Hammerstad and Cutler 1970) If the sink action is of physiological importance for brain function one would expect that this mechanism also operates from spinal cord into spinal CSF However our present knowledge about transport mechanisms in spinal subarachnoid space is sparse No structures with histological characteristics of actively transporting tissues like the choroid plexa are found in the spinal subarachnoid space In spite of this a carrier mediated transport out of spinal CSF has been reported for iodide (Hammerstad Lorenzo and Cutler 1969 Coben and Smith 1969) glycine (Murray and Cutler 1970) and 5 hydroxyindoleacetic acid (5 HIAA) (Bulat and Zivkovic 1971) In the present investigation our earlier studies on the clearance of quaternary amines from ventricular CSF (Aquilonius and Winbladh 1972) are extended to the spinal compartments of the subarachnoid space

Methods

23 adult rabbits (Small Chinchilla) weighing 2.5–3 5 kg were used The animals were anesthetized with halothane 2–2 5 in a mixture of oxygen–nitruos oxide (1 2) and allowed to respirate freely Rectal temperature was kept at 38 C, respiration was registered with a pressure transducer (Statham PT 5 A) connected to a polygraph (Grass Instruments Inc) and in some experiments blood pressure in the femoral artery was recorded with a Statham P 3AC transducer The spinal subarachnoid space was perfused with artificial CSF by three different routes

1 Lumbocisternal (L_{5-6}-cisterna magna)
2 Thoracocisternal (Th_{3-4}-cisterna magna)
3 Lumbothoracic (L_{5-6}-Th_{4-5})

Access to the subarachnoid space was gained by introducing a fine polyethylene tube (PE 50 Intramedic) through a fine slit in the dura meninx after partial laminectomy on one or two vertebrae The operating wound was filled with paraffin oil Backward leakage through the slit in the dura was usually minimal A constant infusion rate of 30 60 or 90 μl/min was obtained with a syringe pump (Model 355 Sage Instruments) The effluent was drained with a roller pump (Stålprodukter Uppsala) with a rate of 30 μl/min when infusion rate was 30 or 60 μl/min and 60 μl/min when infusion rate was 90 μl min. The effluent was collected in 5 minute samples with an automatic fraction sampler In addition 2 perfusions were performed from the lateral ventricle to the cisterna magna as described elsewhere (Aquilonius and Winbladh 1972) *In vitro* studies of choroid plexa were performed as described earlier (Eriksson and Winbladh 1971)

The following labelled drugs were used methyl ^{3}H-decamethonium, 3 9 Ci mol and methyl ^{3}H-choline 15 Ci mmol (Radiochemical Centre Amersham England) ^{3}H methylatropine 430 Ci mol (synthesized from ^{3}H atropine as described by Winbladh 1972) and ^{14}C-carboxyl inulin 3 54 mCi g (NEN Corp USA) The inulin was added as a marker of the dilution of the effluent by original CSF

Composition of the CSF buffer assay of radioactivity and calculation principles have been described earlier (Aquilonius and Winbladh 1972)

After the perfusions the spinal cord was immediately dissected out and homogenized in a Potter Elvehjem homogenizer One part of the homogenate was analyzed for its radioactivity content by oxygen combustion as described elsewhere (Eriksson and Winbladh 1971) Another part of the homogenate was extracted in 7 trichloracetic acid (TCA) (1 3 by volume) centrifuged (5 000 g for 4 min) and the pellet washed 3 times with 1 2 of the same solution In the homogenates from the *choline* perfusions the pellet was thereafter extracted with three volumes of ethanol chloroform (1 1) centrifugated (5 000 g for 4 min) and the pellet washed 3 times with the same solution The radioactivity content of the supernatants from these two extraction procedures as well as the remaining pellets were determined by liquid scintillation the latter after oxygen combustion Less than 5 of the total radioactivity was found in the pellet *after the last extraction Spinal cord extracts and effluent samples from some* experiments were submitted to high voltage paper electrophoresis in a pyridine–acetate buffer (pH 4 6) as described elsewhere (Aquilonius and Winbladh 1972) The total recovery of the spotted radioactivity was about 85 per cent with this

TABLE I Percentual elimination of drugs[a] from spinal perfusates at different infusate concentrations

Drug	Perfusion rate µl/min	Lumbocisternal		Thoracocisternal		Lumbothoracic	
		5 µM	0 1 mM	5 µM	0 1 mM	5 µM	0 1 mM
Choline	30	99	96	68	60	73	57
		97	3ʾ			95	86
	60	52	34*			71	38*
Methylatropine	30	46	39	46	39	31	31
						50	49
						47	40
	60	92	75*			56	54
		92	76				
Decamethonium	30	37	33				
		36	36				
	60	22	28				

[a] Mean value of steady state
Significantly lower than elimination at 5 µM $p < 0\ 01$

procedure The TCA was removed with ether before electrophoresis The electropherograms were scanned in a Packard radiochromatograph and the radioactivity content of the peaks determined by elution or combustion and liquid scintillation. With this method metabolites constituting more than 2-3 of extract radioactivity can be detected

Results

The experimental procedure did not influence respiration blood pressure or gross appearance of the animals Both ^3H and ^{14}C concentrations in the effluent usually reached plateaus within 20 50 min However when perfusion rate was 30 µl/min the establishment of stable outflow concentrations was sometimes so slow that it was difficult to investigate concentration dependance of the elimination from the perfusate In such experiments the perfusion rate was usually changed to 60 µl/min In some perfusions trypanblue was added to the perfusate at the end of the experiment The colour seemed to be rather evenly distributed around the spinal cord along the perfusion route

Concentration dependence of elimination

In the case of choline the fraction of the drug eliminated from the perfusate decreased with increased inflow concentration in the lumbocisternal as well as the lumbothoracic and thoracocisternal perfusions (Table I Fig 1) With methylatropine such a concentration dependent elimination was found only in the perfusions including the cisterna magna i e it was not found in the lumbothoracic perfusions (Table I Fig. 1) With both drugs a larger fraction of the drug was eliminated in the lumbocisternal than in the lumbothoracic and thoracocisternal perfusions (Table I) The elimination of decamethonium from the perfusate in the lumbocisternal perfusions was only about one third of that of the other two drugs

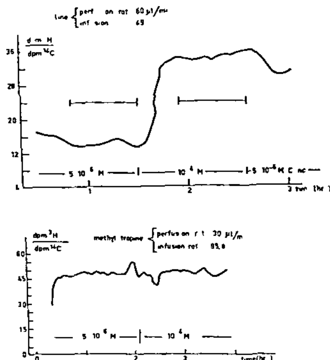


Fig 1 Lumbothoracic perfusion with ³H-choline (upper curve) and H methylatropine (lower curve) in 2 rabbits |—| depicts curve parts used for calculation of clearance x axis time and concentration of drug in the perfusate y axis ratio between drug radioactivity (dpm H) and inulin radioactivity (dpm ¹⁴C)

and no concentration dependence was found (Table I) From Table I it is also seen that the elimination from the perfusate in the lumbocisternal perfusions is inversely related to the perfusion rate

Since only choline was eliminated from the lumbothoracic perfusions by a concentration dependent elimination we made a further investigation of the nature of this uptake In two ventriculocisternal perfusions the elimination of choline decreased significantly on addition of amphetamine 0 1 mM to the perfusate However no such influence was found in the lumbocisternal perfusions (Table II) *In vitro* experiments on rabbit choroid plexa were thus performed as described elsewhere (Eriksson and Winbladh 1971) Amphetamine (0 1 mM) inhibited the uptake of choline (5 μM) in this tissue by 85 per cent (S D = 10 n = 4) In addition decamethonium did not influence choline elimination in one lumbothoracic perfusion in contrast to the finding with ventriculocisternal perfusions (Aquilonius and Winbladh 1972) Further decamethonium inhibits choline uptake in isolated choroid plexus (Eriksson and Winbladh 1971)

Organ concentrations of infused drugs

Choline penetrated into the spinal cord considerably more than the other two drugs while inulin was not found in significant amounts Table III gives total amount of radioactivity recovered from spinal cord as a fraction of total amount infused in the lumbocisternal perfusions

TABLE II Influence of amphetamine on clearance of choline from ventriculocisternal and lumbocisternal perfusates

Route	Perfusion rate µl/min	Percentual elimination[a]	
		Choline 5 µM	Choline 5 µM with Amphetamine 0 1 mM
Ventriculo cisternal	30	77 (85)	66
		89	82
Lumbo cisternal	30	61	61
		70	71
	60	80	81

[a] Mean steady state elimination
Significantly lower than elimination without amphetamine $p < 0.01$
( ) Elimination of choline after amphetamine period

In the lumbocisternal perfusions the choroid plexus of the fourth ventricle contained a higher concentration of radioactivity than that in the last effluent sample In the lumbo-thoracic perfusions no significant radioactivity was found in 0 3 ml of cisternal CSF drawn at the end of the experiments or in fourth ventricle choroid plexa

Metabolic transformations of infused drugs

After the perfusions with decamethonium and methylatropine neither effluents nor spinal cord extracts contained any metabolites according to the electropherograms In contrast after the choline perfusions a considerable proportion of the recovered radioactivity was in the form of metabolites both in the spinal cord extracts and in the last perfusion effluents (Table IV) About half of the radioactivity in the spinal cord homogenates was found in *the TCA extract* (50 ± 20 %, $n = 4$) *and the rest in the chloroform-methanol extract* (phospholipids) In the TCA-extract around 90 % of the radioactivity migrated in one peak as phosphorylcholine and betaine in electrophoresis at pH 4 6 With electrophoresis at pH 10 this peak separated into a larger peak migrating as phosphorylcholine and a smaller as betaine (Table IV) A detailed description of the separation procedure has recently been published (Sparf 1973) The same metabolic pattern after electrophoresis at pH 4 6 was found in the last perfusion effluents although the major fraction was found in the choline peak (Table IV) In 2 expts the spinal cord was frozen *in situ* with liquid nitrogen in the living animal after laminectomy (Th_{4-11}) No labelled acetylcholine could be detected in these TCA extracts when investigated with electrophoresis at pH 4 6

Discussion

Choline is eliminated from the perfusate partly by a concentration dependent mechanism in both perfused segments of the spinal subarachnoid space Methylatropine on the other

TABLE III Fraction of infused radioactivity recovered from spinal cord in lumbocisternal perfusions Number of examined cases in brackets

Perfusion drug	*Per cent* Mean ± S E.	Perfusion time h
Choline	16 ± 2 (5)	3.5–4 5
Methylatropine	3 3 ± 0 6 (3)	.5–4 5
Decamethonium	? 2 ± 0 3 ( )	4

hand was eliminated by such a mechanism only in those perfusions which included the cisterna magna *i e* lumbocisternal and thoracocisternal perfusions In some species such as dogs and humans tufts of the choroid plexus of the fourth ventricle often project out into the cisterna magna (Davson 1967) We have found no reports concerning this anatomy in the rabbit However the high concentrations of radioactivity found in the choroid plexa in the perfusions including the cisterna magna show that the fourth ventricle choroid plexus somehow gets in contact with the perfusate It thus appears possible that the concentration dependant elimination in these perfusions is mediated by this choroid plexus although another site of transport cannot be excluded The question then rises why the elimination of decamethonium showed no concentration dependance in the lumbocisternal perfusions The most probable explanation to this finding is the low transport capacity of rabbit choroid plexa for this amine found both *in vivo* and *in vitro* (Aquilonius and Winbladh 1972) A support of this explanation is the finding that the elimination of decamethonium in the lumbocisternal perfusions was only about one third of that of choline and methylatropine

The radioactivity content of the spinal cord was considerably higher after the choline perfusions than after the other two drugs This finding suggests that the concentration dependant elimination of choline found in the lumbothoracic perfusion might be due to an uptake of the drug in the spinal cord Whether the concentration dependence is due to an actual active transport of choline into the spinal cord or to the demonstrated enzyme mediated metabolism of choline within this tissue cannot be stated from the present experiments Thus a comparison of this uptake with other reports of uptake of choline in erythrocytes brain slices and synaptosomes does not appear meaningful Anyhow the elimination mechanisms appear to be different in the ventriculocisternal and the lumbothoracic perfu

TABLE IV Fraction of metabol tes (per cent of recovered) [a]

Perfused drug	Perfusate	Spinal cord
Choline	4 I.	88 ± 6 (5)[b]
Methylatrop ne	0 (2)	0 ( )
Decamethonium	0 ( )	0 ( )

[a] High voltage electrophoresis pH 4 6
[b] High voltage electrophoresis pH 10 of e tracts from 2 expts

Phosphorylcholine 77 83
Lecithine 4 5
Beta ne 13 I.
(n) = number of expts

sions since in the former but not in the latter the elimination was decreased by amphetamine The site of the amphetamine sensitive elimination mechanism was probably the choroid plexa since the choroid plexus uptake of choline *in vitro* was partly inhibited by amphetamine This inhibition probably is due to a competition of amphetamine with choline for the choline carrier as shown for other primary and quaternary amines (Tochino and Schanker 1965)

In conclusion it appears as there is no active removal of quaternary amines in general from spinal CSF like that from ventricular CSF It cannot be excluded that the substances, which by others have been claimed to be actively removed from spinal CSF *i e* iodide glycine and 5 HIAA are taken up by the spinal cord itself as shown for choline in this paper Thus a sink action of the CSF for quaternary amines in the case of the spinal subarachnoid space can only be due to bulk flow of CSF However nothing is known about the role of the central canal and its surrounding structures in this respect

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Acta physiol scand. 1975 93 385–390

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Brain Carbonic Acid Acidosis after Acetazolamide

By

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Received 4 September 1974

Abstract

HEUSER D J ASTRUP N A LASSEN and E BETZ *Brain carbonic acid acidosis after acetazolamide* Acta physiol scand 1975 93 385–390

In cats in barbiturate anesthesia extracellular pH and potassium were continuously recorded from brain cortex by implanted microelectrodes Implantation of the electrodes preserved the low permeability of the blood-brain barrier to HCO_3^- and H ions as indicated by the development of brain *acidosis* by i v injection of HCO_3^- Acetazolamide (5 mg/kg) i v was followed by a marked brain acidosis which after 10 min had progressed to a drop in pH of 0.203 ± 0.046 ($\bar{x} \pm$ S D., n = 8) The slowness of the development of acidosis points to a direct effect of the carbonic anhydrase inhibition on the brain tissue As a further support for this conclusion was considered the finding of a prolonged responsetime of brain pH to HCO_3^- i v to CO_2 inhalation and to hyperventilation after the acetazolamide inhibition No changes in brain extracellular potassium were found

Key words Acid–base equilibrium, acidosis acetazolamide brain carbonic acid carbonic anhydrase, carbonic anhydrase inhibitors cerebral cortex, cerebrovascular circulation diamox microelectrodes potassium

I v injection of acetazolamide (diamox) in doses that practically block red cell carbonic anhydrase causes a decrease in cerebral spinal fluid (CSF) pH as measured directly on the brain surface by Severinghaus and Cotev (1968) This acidosis agrees well with the marked increase in cerebral blood flow seen with this drug (Ehrenreich *et al* 1961 Cotev *et al* 1968) since brain extracellular acidosis is well known to be a very strong vasodilator stimulus (Betz and Heuser 1967 Lassen 1969) However more recently Kjallquist Nardini and Siesjo (1969) studying the effects of acetazolamide on the brain tissue found evidence interpreted to indicate that CSF pH remains unchanged and they left open the question of intracellular pH effects in brain

In the present study the development of a marked acidosis of brain extracellular fluid after acetazolamide was confirmed by direct measurements using a specially designed micro-pH glass-electrode implanted in the cortical tissue approx 0.5 mm below the brain surface Implantation of the pH-electrode in the cortex did not influence the low permeability of

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the blood-brain barrier (BBB) to H^+ and HCO_3^- ions as demonstrated by injection of HCO_3^- The pH response following acetazolamide was biphasic possibly due to a dual action of the drug, ri both on the blood and on the brain itself The results support the concept of Severinghaus and Cotev (1968) that a carbonic acid acidosis is produced by a direct acetazolamide action on the brain tissue

Since brain acidosis after acetazolamide has been questioned extracellular potassium in brain known as a vasodilator (Kuschinsky *et al* 1972) was measured along with pH but no changes were found

Methods

Cats in Nembutal® (25 mg kg) anesthesia immobilized using Flaxedil® (1 2,3 tri-(β-triethylammonium-ethoxy)-benzoltrijodid] (3 mg/kg) were artificially ventilated Alveolar CO_2 (vol) was monitored continuously and ventilation adjusted to give normal values of arterial pH and pCO_2 Mean arterial blood pressure (MBP) was recorded from the femoral artery The suprasylvian gyrus on one side was exposed by removing approx 1 cm² of the skull Dura was cut open (2-3 mm²) using a sharp needle This was completed without bleeding from cortical vessels Care was taken to prevent heating of the tissue by water-cooling of the skull-drill by completing the preparation and mounting the electrodes without use of the close up lamps mounted on the stereo-microscope

A potassium microelectrode a pH microelectrode and 2 reference microelectrodes were all mounted through the hole in the dura as close to the ertical position as practically possible The tips of the pH and the K^+-electrodes were implanted about 0 5 1 0 mm down in the brain tissue while tips of the reference electrodes were placed on the brain surface In spite of the small movements of the brain surface synchronous to respiration mounting and the positioning of the electrodes in the brain tissue did not cause bleeding from cortical vessels

The potentials of the potassium-electrode and the pH-electrode were recorded against the same reference electrode The other reference-electrode was grounded and the potential between the 2 reference-electrodes recorded This way potential changes picked up by the reference-electrode and not by the pH and potassium- electrodes were recognized as reference potential changes and could be subtracted the pH and potassium signals. DC amplifiers with high impedance differential input were used

The pH microelectrodes were made according to Gebert (197) of H -selective glass pulled out to form a capillary with an outer diameter of approx 60 μm Except for approx 100 μm of the end the cap llary was sealed on the outside by melting on a larger capillary made of non H^+-selective glass By heating and simultaneously applying a pressure inside the H -selective glass-capillary the open and free end was closed while forming a tiny glass-"bubble" with an outer diameter of approx. 60 μm When f lled with 0 1 N HCl th s glass-"bubble" formed the H -sensitive part of the pH microelectrode The K microelectrodes were made accord ng to Walker (1971) Glass pipettes were pulled out to a tip diameter of about 4 μm. The tip was made hydrophobic by treatment with 3 n butyl-chloro-silane and f lled with K -selecti e liquid ion-exchanger (Corning Code 477317) To prevent the ion exchanger from leaking out the very end of the tip was closed" with 2 agar in mock CSF or 150 mmol NaCl This way the life span for the electrodes increased and th s could be done without increasing the electrode time constant seriously (Heuser unpubl hed) The glass pipette was then f lled with 0.5 N KCl from behind Both the pH microelectrodes and the K -electrodes responded with a potential change of about 53 mV per decade-change in H and K -concentrat on With K -concentrations below 5 mmol 1 the potassium potentials were influenced by Na (150 mmol l) in accordance with a Na -selecti ity against K of about 0 01 The K microelectrodes were not sen iti e to H in concentration correspond ng to pH from 6 to 8 The electrodes were calibrated at room temperat re immediately before implantat on in the bra n Glass pipettes with a tip-diameter of about 20 μm and f lled w th 0 9 NaCl in 1 agar served as reference-microelectrodes.

Results

HCO_3 injected i v leads to blood alkalosis and to liberation of CO_2 In the brain the response is an acidosis since the intact BBB is practically impermeable to H and to HCO

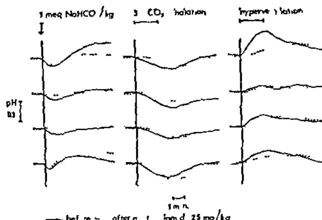


Fig 1 Responsiveness of brain pH to 1 meq $NaHCO_3$/kg i v to 5 CO_2 inhalation and to hyperventila tion The pH changes develop more slowly after acetazolamide inhibition Note that HCO injection causes brain acidosis, indicating preserved low permeab lity of the blood–brain barrier to HCO_3 and H^+ around the pH electrode implanted in the brain tissue

but allows CO_2 as gas to diffuse into the brain Experimentally the preserved low permea bility of the BBB to HCO_3^- and H ions was demonstrated as an acidosis recorded by the pH-electrode following injection of HCO_3^- (1 meq/l) i v This was tested before and after acetazolamide (Fig 1) In all cases an acidosis developed This indicates that the pH-elec trodes implanted in the brain tissue measure pH *in brain* essentially unaffected by blood pH This test was considered a prerequisite for the acetazolamide experiments

Acetazolamide 25 mg/kg i v was followed by a marked brain tissue acidosis (Fig. 2) After 10 min pH had decreased by 0 203 ±0 046 units (n = 8) (Table I)

Before the acidosis all experiments showed a brief biphasic alkalotic swing in the pH signal (Fig 2) This will be commented on in the discussion

The responsiveness of brain tissue pH to rapid changes in arterial pCO_2 is shown in Fig 1 and Table II Time to peak of the changes in brain tissue pH following HCO_3 1 meq/ kg i v inhalation of CO_2 5 vol and hyperventilation is longer after acetazolamid (approx 20–30 min after the injection of acetazolamid) (Table II) This indicates a slower absorp-

TABLE I Brain pH and brain potassium (mmol/l) recorded init ally after implantation of the electrodes and before and 10 m nutes after acetazolamide 25 mg/kg i v A marked bra n a idosis de eloped without detectable cha ges in brain extracellular fluid potass um. No relat on was found between init al pH and in tial potassium concentration

Brain pH x±S D (n = 8)			Bra n potassium (mmol/l) x±S D (n = 8)			ΔCO vol
Initial measure ment	Before acetazol am d	ΔpH	In tial measure ment	Before acetazol amid	ΔK+	
7 37 ±0.1	7 3 ±0 1	−0 20 ±0 05	6 0 ±.8	6 3 ±3 3	0 1 ±0 3	−1 0 ±0 2

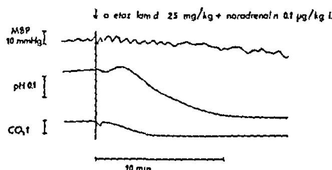


Fig 2 Brain pH mean arterial blood pressure (MBP) and alveolar CO_2 (vol) following i v injection of acetazolamide 25 mg/kg mixed with noradrenaline 0 1 µg/kg No blood pressure drop occurred Note the alkalotic swing in the pH signal with maximum 2 min after the injection followed by the marked acidosis

tion and release of CO_2 by the reaction



$$[H^+ + HCO_3^- \rightleftharpoons H_2CO_3] \rightleftharpoons H_2O + CO_2$$

and thus points to a direct effect of acetazolamide in brain tissue

Initial values of pH and K^+ mmol/l in brain tissue obtained immediately upon electrode implantation are shown in Table I No relation between the 2 variables was found Acetazolamide did not cause any detectable shift in brain potassium It is then excluded that extracellular potassium adds to the increase in cerebral blood flow seen with this drug

Acetazolamide in form of diamox® is obtained as the sodium salt of acetazolamide which in aqueous solution has pH of about 9 5 As a control to the injection of acetazolamide sodium we titrated the solution with 0 1 N HCl to pH 7 4 and injected the equivalent amount of NaOH (0 16 meq/kg) before and after acetazolamide resulting in a rapid decrease in alveolar pCO_2 and a brain alkalosis of about 0 02–0 05 pH units

Discussion

The reproducible rapid alkalotic swing seen initially in the pH signal following acetazolamide injection (Fig 2) cannot be secondary to H^+ and HCO_3^- changes in the blood since

TABLE II Time for peak in brain pH changes following intravenous injection of 1 meq $NaHCO_3$ kg, inhalation of 5 % CO_2 and hyperventilation and changes in alveolar CO_2 (vol %) after 2 min of inhalation of 5 % CO_2 and of hyperventilation Before and after acetazolamide 25 mg kg i v

Acetazolamide 25 mg kg i v	Time (min) for peak pH			Alveolar CO_2 vol (after 2 min)	
	HCO_3 1 meq kg i	5 % CO_2 inhalation	Hyperventilation	5 % CO_2 inhalation	Hyperventilation
Before $\bar{x} \pm$ S D	0 84 ± 0 34 n = 5	75 ± 0 47 n = 6	2 0 ± 0 7 n = 5	5 16 ± 0 17 n = 7	1 69 ± 0 n = 7
After $\bar{x} \pm$ S D	0 9 ± 0 7 n = 5	1 45 ± 0 45 n = 6	3 55 ± 0 38 n = 4	5 06 ± 0 39 n = 7	1 17 ± 0 15 n = 7
p	0 35	0 001	< 0 001	< 0 77	0 001

the BBB was shown to be intact with respect to these ions (Fig. 1). Immediately after the acetazolamide injection and until brain carbonic acid anhydrase has become significantly inhibited pH changes in the brain as measured by the implanted pH-electrode can be assumed to reflect pCO_2 changes in the intoxicated circulating blood. CO_2 from the blood diffusing into the brain will almost instantly be equilibrated according to the reaction scheme $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$ which at that time is still catalyzed by brain carbonic anhydrase. We might then attempt to explain the alkalotic swing in the pH-curves (Fig. 2) as caused by a decrease in arterial pCO_2 following acetazolamide intoxication. According to Maren (1967) carbonic acid is retained in the blood and the reaction above not equilibrated rapidly enough for the blood to release sufficient amounts of CO_2 in the lungs. Acetazolamide increases total blood carbonic acid and decreases arterial pH, alveolar pCO and hence end pulmonary capillary pCO_2 decreases markedly with arterial pCO_2 steadily rising although remaining lower than normal in the blood reaching the tissues; on the other hand pCO_2 in sampled arterial blood is slightly higher than normal when measured after equilibrium has been reached. The observed alkalotic swing in brain pH might thus be explained by hypocapnia in the circulating arterial blood. The alkalosis during the first minute is converted into an acidosis along with the progressive acetazolamide intoxication of brain.

The main finding of this study is the marked brain acidosis which develops rather slowly after the acetazolamide injection and after 10 min corresponds to a drop in pH of 0.203 ± 0.046 units as recorded extracellularly in the brain cortex.

Effective carbonic anhydrase inhibition of the red cell by acetazolamide occurs within the first minute after administration of the drug (Maren *et al.* 1961). The decrease in brain pH develops more slowly and this points to carbonic acid anhydrase inhibition in the brain tissue being retarded by the more slow penetration of the drug across the blood–brain barrier (Roth *et al.* 1959; Rall *et al.* 1962; Travis *et al.* 1966; Maren 1967).

This interpretation is in consonance with that of Severinghaus and Cotev (1968) who found CSF acidosis even when CSF pCO_2 was maintained constant. This shows that a steady state of disequilibrium of the $[H^+ + HCO_3^- \rightleftharpoons H_2CO_3] \rightleftharpoons CO_2 + H_2O$ system is produced. This precludes the use of the Henderson Hasselbalch equation for indirect pH measurement using the pCO_2–HCO_3^- system. Accordingly the lack of CSF acidosis as estimated in this indirect manner by Kjällquist *et al.* (1969) may be disregarded.

The pH inside the brain cells is probably also shifted to the acid direction by acetazolamide as the decrease in brain tissue lactate and pyruvate seen with acetazolamide corresponds to that seen in tissue acidosis produced by hypercapnia (Kjällquist *et al.* 1969; Schindler *et al.* 1973). The acid responsible is in all probability carbonic acid. This conclusion is supported by the evidence of a disequilibrium in the CSF mentioned above. And with regard to the intracellular fluid compartment further support is afforded by the finding
an increase of intracellular bicarbonate concentration (Kjällquist *et al.* 1969). The
taneous increase in HCO_3 and in H^+ concentration and hence of H_2CO_3 is
is required in the steady state to produce CO_2 at unchanged rate if tissue anhydrase is inhibited. If CO were the end product of oxydative metabolism in the
amide would have no effect on pH if pCO_2 was kept constant. T

presence of carbonic anhydrase in the brain cells (Giacobini 1962) suggest that in brain carbonic acid (H_2CO_3) and not its anhydride (CO_2) is the end product of oxydative de carboxylation as pointed out by Severinghaus and Cotev (1968) and by Severinghaus Hamilton and Cotev (1969) Why their elegant and convincing experiments and arguments have become so little recognized in current descriptions of this fundamental problem is difficult to understand A thorough discussion of the problem lies however outside the scope of this communication that merely aims at stressing that extracellular acidosis in brain is indeed very marked after acetazolamide and that this points to carbonic acid acidosis in brain both of the extra and intracellular compartments

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Acta physiol scand 1975 93 391–400
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The Metabolism of Acetate in the Perfused Hind Quarter of the Rat

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Received 10 September 1974

Abstract

Karlsson N E Fellenius and K H Kiessling *The metabolism of acetate in the perfused hind-quarter of the rat* Acta physiol scand 1975 *93* 391–400

The elimination kinetics of acetate the main end product of ethanol metabolism in the liver and the influence of acetate oxidation on the redox and energy state of the isolated perfused hind quarter of the rat were studied The rate of acetate uptake increased with increasing initial concentration of acetate in the perfusion medium suggesting that the plasma level of free acetate may be one factor in the regulation of acetate uptake in the skeletal muscle Addition of acetate as a single dose did not affect the net production of lactate or the uptake of glucose In continuous infusion experiments at a constant concentrat on of ? mM of acetate in the medium the lactate/pyruvate ratio was unaffected in the medium and in the muscle tissue Addition of acetate did not affect the oxygen uptake Experiments with ^{14}C acetate showed that about 50 % of added radioactivity was found in form of $^{14}CO_2$ accounting for 25 to 45 of the oxidative metabolism in the muscle tissue It was calculated that about 5 of the acetate produced in the liver during ethanol oxidation can be consumed in the resting perfused hind quarter of the rat The t ssue conte t of high-energy phosphate compounds was not s gnificantly affected by acetate
K y w d acetate metabolism, skeletal muscle perfused h n}-quarter

During hepatic ethanol oxidation acetate is formed stoichiometrically (Lundquist *et al* 1962) and 70–80 % of the oxidized ethanol appears as free acetate in the hepatic venous outflow (Fellenius *et al* 1973 a Damgaard *et al* 1973) Due to the further rapid oxidation of acetate in extrahepatic tissues (Lundquist *et al* 1962 Forsander *et al* 1960 Lindeneg *et al* 1964) low levels of acetate are found in the peripheral circulation The metabolism of acetate in different tissues and in whole animals has been investigated by several authors (Annison 1971 Ballard 1972 Dhopeshwarkar *et al* 1971 Freundt 1968 Freundt 1973 Ghosal *et al* 1969 and Smyth 1947) but relatively little attention has been paid to skeletal muscle It has been shown (Aas 1971) that the myocardium, in contrast to the skeletal muscle has a very high activity of acetate thiokinase (E E 6.2 1 1) which is the rate limiting enzyme in the metabolism of acetate (Barth *et al* 1972 Knowles *et al* 1974) It is however evident from investigations by Forsander *et al* (1960) and Lundquist *et al* (1973) that since the skeletal muscle accounts for about 40 % of the body weight in most mammals this tissue will be of importance in the metabolism of acetate

The purpose of this investigation was to study 1) the capacity of the isolated perfused hind-quarter of the rat to utilize acetate 2) the elimination kinetics of acetate 3) the metabolic fate of acetate and 4) the influence of acetate on the redox and energy state of the skeletal muscle

Materials and Methods

Female Sprague Dawley rats weighing 200–260 g, purchased from Anticimex, Sollentuna Sweden were used. The rats were fed *ad libitum* on the standard small-animal diet (Astra Ewos, Södertälje S eden) and had free access to water prior to the experiments.

Standard analytical grade laboratory reagents were obtained from E. Merck AG Darmstadt, West Germany Enzymes and co-enzymes were obtained from Biochimica Boehringer Mannheim, West Germany or Sigma Co St. Louis USA Solutions of hexobarbital (Evipan® Sodium Bayer AG Leverkusen, West Germany) (100 mg ml) were freshly prepared from hexobarbital powder Bovine serum albumin (fraction V) was obtained from Pentex Corp (Miles Laboratory Inc Kankakee Ill USA) Stock solutions of albumin (10 %) were prepared in Krebs-Henseleit high bicarbonate buffer (Krebs and Henseleit 193_) and dialyzed against the same buffer

U-^{14}C acetate (58 5 mCi mmol) was obtained from Radiochemical Center Amersham England Instagel and ethanolamine was purchased from Packard Instrument Co Inc. Ill USA 2,5-diphenyloxazole (PPO) and 1 4-di-(_-(5-phenyloxazolyl)-) benzene (POPOP) from Koch Light Laboratories Ltd Colnbrook Buckinghamshire England Phentolamine was a gift from Hässle-CIBA-Geigy Gothenburg, Sweden

Perfusion technique and experimental procedure

The method of rat hind-quarter perfusion used was that described by Ruderman *et al* (1971) All perfusions were performed on skinned animals with resting hind legs. The perfusion medium was composed of Krebs-Henseleit high bicarbonate buffer containing 4 % bovine albumin fresh and washed bovine red cells (7–8 g hemoglobin 100 ml) 5.5 mM glucose and 0 10 mM pyruvate. Phentolamine (0 10 mg ml) was added to prevent vasoconstriction. The bovine red cells were washed four times in the high bicarbonate buffer before use The final volume of the medium was 150 ml The medium was gassed in a multibulb oxygenator with O_2 + CO_2 (95 5) The initial concentration of lactate in the medium was about 1 mM Glucose consumption and lactate production due to glycolysis in the red cells and hemolysis of the red cells were negligible

The rats were anesthetized by intra-peritoneal injections of hexobarbital (25 mg 100 g b wt) The first 5 ml of the medium were discarded and the medium was then recycled A "zero"-sample was taken at 10 min before the addition of acetate Sodium acetate dissolved in H_2O was added either as a single dose or by continuous infusion after a priming dose of sodium acetate Samples for analysis of acetate in the single dose experiments were taken at 15 20 25 35 45 and 55 min The disappearance of acetate was calculated from the linear part of the progressing curves Samples for analysis of acetate in the infusion experiments were taken at 15 _5 40 55 and 70 min Samples from the medium for analysis of lactate pyruvate and glucose were usually taken at 10 and 55 or 70 min respectively

Analytical methods

Samples of the medium for analysis of the concentration of acetate were added to _ M $HClO_4$ After centrifugation the amount of acetate in the supernatant was determined by the microdiffusion method of Seri_ and Cotzias (1955) as modified by Keane (19_7) The oxygen content of the medium was determined with an oxygen-electrode according to Solymar *et al* (1971) as modified by Dr Tottmar in our laboratory (unpubl. res.) Oxygen consumption was calculated from arterio-venous differences of O_2-content and flow rate The flow rate was determined from the number of drops falling into the reservoir Samples for analysis of lactate pyruvate and glucose were deproteinized in ice-cooled 0 6 M $HClO_4$ The extracts were neutralized with KOH and the precipitated $KClO_4$ was removed by centrifugation The concentrations of lactate and pyruvate were measured in these extracts as described by Hohorst *et al* (1959) The amount of glucose was determined enzymatically as described by Bergmeyer (19_0)

For the analysis of muscle tissue a portion of the thigh muscles was rapidly frozen in a _ with aluminum clamps cooled in liquid nitrogen (Wollenberger *et al* 1960) The frozen tissue was grounded in a mortar cooled in liquid nitrogen and was homogenized in ice-cooled 0 6 M $HClO_4$, after which it was

treated as described above The amount of adenosine triphosphate (ATP) was determined according to Lamprecht and Trautschold (1962) adenosine diphosphate (ADP) and adenosine monophosphate (AMP) according to Adam (196) and creatine phosphate (CP) by the method of Lamprecht and Stein (196)

Isotope measurements

The metabol c fate of acetate was determined in three separate experiments with ^{14}C-labelled acetate The experiments were performed like the infusion experiments with non labelled acetate except that U ^{14}C acetate was added to give a specific activity of 9 nCi μmol of acetate both in the priming dose and the infusion solution

Determination of $NaH^{14}CO$ in the medium was performed according to Wolner *et al* (1973) $^{14}CO_2$-gas was collected by connecting the gas-outlet from the oxygenator and the space above the medium in the reservoir to a bottle containing 40 ml of ethanolamine/ethylene glycol monomethyl ether (1 2 v/v) The collection was accompl shed by connecting the system to a water aspirator 1 ml of the ethanolamine/ethylene glycol monomethyl ether solution was added to 5 ml ethanolamine/ethylene glycol monomethyl ether/toluene (1 7 10 v/v/v) containing 5 5 g per liter of 2 5-diphenyloxazole (PPO) for liquid scintillation counting (Jeffay and Alvarez 1961)

Lipids in the muscle tissue were extracted overnight in chloroform/methanol (1 v/v) and incorporation of label was measured according to Holm and Schersten (1972) Measurements of radioactivity in water soluble substances in the muscle tissue was done by counting in Instagel an aliqout of the perchloric acid extract obtained as described above

Radioactivity was measured in a Nuclear Chicago Mark II l quid scintillation counter

Expr ssion of results

Results are expressed as mean$\pm$S E with the number of observat ons in parentheses unless otherwise stated Students t test was used for statistical analysis

Thanks are due to Mrs Å. Paul n and Miss G B Jönsson for skilful technical ass stance and to Mrs E Nises Ahlgren for typing the manuscript The authors are indebted to Dr O Tottmar for provision of the oxygen electrode apparatus

Results

To determine the total mass of the perfused muscle tissue Evans Blue was injected into the arterial circulation of a number of rats at the end of the perfusion The muscle was there after handled as described by Ruderman *et al* (1971) By using this technique a correlation between body weight and the weight of the perfused muscle tissue was obtained as shown in Fig 1 The correlation coefficient of 0 95 is statistically significant ($p < 0\,001$) This dia gram was used to calculate the total weight of the perfused muscle in the experiments where a portion of the muscle was freeze-clamped

The rate of disappearance of acetate from the medium as a function of the initial con centration of acetate is shown in Fig 2 It was found that the utilization of acetate increased with increasing initial concentration of acetate in the medium The correlation coefficient of 0 89 is statistically significant ($p < 0\,001$)

The glucose uptake (Fig 3) was not affected by the addition of acetate whereas a low initial concentration of acetate (1 mM) slightly inhibited the net production of lactate The lower level of lactate in this experiment as compared to the control caused the lactate/pyruvate ratio to decrease No changes in the lactate/pyruvate ratio were observed in experi ments where higher initial levels of acetate were used

In order to study the utilization of acetate when the substrate was kept at an almost

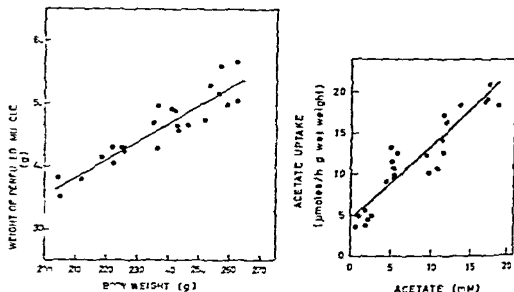


Fig. 1. The relationship between body weight and weight of the perfused muscle tissue of the hind-quarter of the rat.

Fig. 2. The removal of acetate by the resting, perfused hind-quarter of the rat at different initial concentrations of acetate in the perfusion medium. Estimations of acetate uptake have been made from the linear part of the progressing curve during 40 min of perfusion. Each point represents one experiment.

constant level in the medium, thereby imitating the situation during ethanol intake in man and rat *in vivo* (Lundquist *et al.* 1962, Majchrowicz 1973) experiments were performed where acetate was added as a single dose, followed by infusion of the substrate into the medium at the same rate as it disappeared from the medium in the single dose experiments at concentrations of 2 and 5 mM respectively. The latter concentration was chosen to study the effect of a high level of acetate on the metabolism of the skeletal muscle. As can be seen in Table I, the utilization of acetate at a constant concentration of the substrate in the medium is of the same order of magnitude as in the single dose experiments at the corresponding initial concentrations (Fig. 2). The oxygen uptake was found to be constant throughout the experimental period. The addition of acetate did not alter oxygen uptake (Table I).

The incorporation of label into $^{14}CO_2$ accounted for about 50% of the acetate uptake (Table II). A minor portion of radioactivity was found in tissue lipids, while a considerable part of the label was found in water soluble metabolites obtained in the perchloric acid extracts of the muscle. Since the specific activity of ^{14}C-acetate in the medium (cpm/µmol of acetate) did not change during the perfusion it is likely that water-soluble intermediates of acetate did not leave the tissue.

In contrast to the single dose experiments, a steady-state level of 2 mM of acetate did not affect the net production of lactate (Fig. 4). The lactate pyruvate ratio in the medium showed only small changes at both concentrations used. The small changes of the lactate pyruvate ratio were usually reflected by a similar change in the muscle tissue.

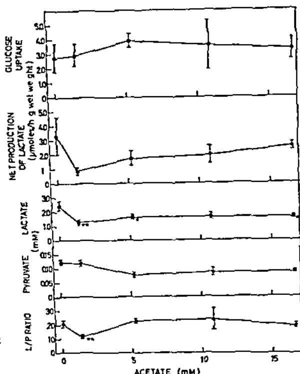


Fig. 3 Influence of different init al con centrations of acetate on glucose uptake net production of lactate the lactate and pyruvate levels and the lactate/pyruvate ratio in the *medium at the end of the* perfusion

Results are expressed as mean ± S E. of 6 5 7 5 and 4 observations at the following acetate le els 0 1 5 10 and 15 mM respectively Acetate was added as a single dose The acetate concentra *tions refer to observed initial levels* $= p < 0.05$ $= p < 0.01$ Significant difference compared to control perfusion

Measurements of the high and low-energy phosphate compounds from freeze-clamped samples of the perfused muscle tissue (Table *III*) *showed that the presence of a constant* level of acetate significantly changed the tissue content of CP and AMP in comparison with the control

Discussion

Elimination of acetate

The utilization of acetate increased with increasing initial concentration of acetate in the medium (Fig 2) In this respect the metabolism of acetate resembles that of long-chain fatty acids (Fritz 1961) A similar correlation between acetate concentrations and the utilization of acetate was found in the acetate infusion experiments (Table I) From these results it is suggested that at the concentrations used in this investigation the plasma level of free acetate may at least be one important factor in the over all regulation of acetate uptake in the resting skeletal muscle Other investigators have come to the same conclusion in studies with the human heart (Lindeneg *et al* 1964) and with the perfused rat heart (Williamson 1964) Similar results were recently obtained in experiments with the working human forearm (Lundquist *et al* 1973) where acetate uptake correlated to the load of acetate offered to the muscle at arterial concentrations of about 1 mM

TABLE I Rate of acetate and oxygen uptake in the perfused resting hind-quarter of the rat at constant levels of acetate in the perfusion medium

Substrate added	Acetate consumption (μmol/h g wet weight)	Oxygen consumption
None	—	24 3±1 9 (6)
Acetate 2 mM + infusion	7 0±0 7 (6)	27 5±0 7 (6)
Acetat 5 mM + infusion	9 8±1 9 (9)	25 7±0 9 (8)

Results are expressed as mean±S E with the number of observations in parentheses Acetate was added as single doses (2 and 5 mM respectively) followed by continuous infusion of acetate at a rate of 7 0 μmol/h g wet weight and 9 0 μmol/h g wet weight The total amount of muscle was obtained from the rat weight and the correlation in Fig 1

Quantitative relationship between acetate production in the perfused rat liver and the uptake of acetate in the perfused hind legs

It has previously been demonstrated (Fellenius *et al* 1973 a) that the perfused liver of a fed rat produces about 100 μmol/h g wet weight of acetate during ethanol oxidation A 220 g rat with a liver weight of 10 g will thus produce acetate at a total rate of 1 000 μmol/h From Fig 1 it can be seen that the hind-quarter muscle weight of a 220 g rat is about 40 g Multiplying this weight by the obtained rate (7 0 μmol/h g wet weight) of acetate elimination at a steady-state concentration of 2 mM of acetate in the medium (Table II) a figure of 280 μmol/h is obtained More than 25% of the acetate produced by the liver during ethanol oxidation can therefore be consumed by the resting skeletal muscles in the hind-quarter Taking into account other skeletal muscles the figure 25% will increase to about 60 (assuming a muscle weight of 40% of the body weight) However other tissues besides the resting skeletal muscles must also contribute in order to keep the amount of acetate at such low levels in the plasma during ethanol metabolism as have been reported in the literature (Lundquist *et al* 1962 Lindeneg *et al* 1964 Majchrowicz 1973)

TABLE II The metaboli fate of U ^{14}C acetate in the perfused hind-quarter of the rat

Substrate added	Acetate consumption (μmol/h g wet weight)	Incorporation of label into CO$_2$ (μmol/h g wet weight)	Lipids	Water soluble metabolites in muscle tissue	Oxygen consumption due to oxidation of acetate to H$_2$O and CO$_2$ ( )
^{14}C acetate [illegible] mM ([illegible]6 μCi) infusion	7 0 (6 9 7 [illegible])	3 5 ([illegible] 4 [illegible])	0 05 (0 04–0 0[illegible])	3 4 (3 [illegible] 3 6)	[illegible]5 2 (17 5 [illegible] 9 8)
^{14}C acetate 5 mM (7 5 μCi) infusion	10 3 (9 9 10 9	5 6 (4 [illegible] 6 7)	0 07 (0 06–0 07)	4 0 (3 4 4 5)	43 6 (36 6 [illegible] [illegible])

Experimental conditions see Table I and Methods The mean value of 3 observations with the highest and lowest value in parentheses

TABLE III Concentrations of adenine nucleotides and creatine phosphate in the muscle tissue of the resting perfused hind quarter of the rat at the end of the experimental period at constant levels of acetate in the perfusion medium

Substrate added	ATP (μmol/g wet weight)	ADP	AMP	CP
None	6 03±0 31 (6)	0 639±0 0_0 (6)	0 013±0 007 (6)	10 3 ±1 3 (6)
Acetate 2 mM + infusion	7 2_±0 67 (6)	0 696±0 027 (6)	<0 005 (6)	14 3 ±1 (6)
Acetate 5 mM + infusion	5 13±0 48 (8)	0 668±0 021 (8)	0 032±0 012 (10)	16 3 ±1 5 (8)

Results are expressed as mean±S E with the number of observations in parentheses Experimental conditions see Table I = $p<0.05$ = $p<0.01$ Significant difference compared to control perfusion

perfused rat heart that acetate changed glucose uptake very little while the incorporation of labelled glucose into glycogen was increased Ruderman *et al* (1971) showed that glucose accounts for a very small part of the oxidative fuel in the perfused rat hind-quarter at rest, most glucose being utilized for glycogen synthesis It is possible that in the present investigation the unaffected glucose uptake in combination with a slightly inhibited net production of lactate (Fig 3) reflects such a stimulation of glycogen synthesis In this connection it is interesting to note that alcoholics usually have a lower activity of skeletal muscle lactate dehydrogenase compared with non alcoholics (Kiessling *et al* 1973) which indicates that the production of lactate is decreased

A low initial level of acetate added as a single dose lowered the lactate/pyruvate ratio in the medium (Fig 3) whereas this ratio was unchanged at the low and constant concentration of acetate (2 mM) (Fig 4) It is evident that the manner of administration of the substrate into the medium is of great importance for the lactate/pyruvate ratio

During the activation of acetate ATP is transformed to AMP and PP One might expect that this reaction would be reflected as an increase in AMP which is normally very low compared to ATP (Fellenius *et al* 1973 b) An increase in the concentration of AMP was also found at the high and steady state concentration of acetate but not at the low level (Table III) thus showing the concentration dependent change in the amount of AMP No significant changes in the content of ATP or ADP were found while the content of CP was elevated indicating that a high level of high-energy phosphate compounds is maintained during acetate metabolism although the combustion of other substrates is depressed

This work was supported financially by Hierta Retzius Foundation Helge Ax son Johnson Foundation AB Helios Kemisk Tekniska Fabriker the Faculty of Mathematics and Science of the University of Uppsala and by Grant B73 I Y 364-06 from the Swedish Medical Research Council.

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Acta physiol scand 1975 93 401–408
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No Role for Zinc in the Storage of Histamine in Rat Peritoneal Mast Cells

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Received 11 September 1974

Abstract

UVNAS B C H ÅBORG and U BERGQVIST *No role for zinc in the storage of histamine in rat peritoneal mast cells* Acta physiol scand 1975 *93* 401–408

The histam ne in the granules of mast cells has been proposed to be stored as a heparin–zinc histamine complex Due to its chelating action zinc should increase (double) the histamine binding capacity of he parin We ha e determined the zinc content of isolated rat peritoneal mast cells to 2.4–4 1 nmol/10^6 cells and of their granules to 13 0–21 4 nmol/mg dry weight The corresponding amounts of histamine were 150 and 680 nmol respectively The zinc content found is far (0 times) too low to allow for an adequate bind ng of h stam ne in a heparin–zinc–histamine complex *In vitro* the granules take up z nc in the same manner as they take up other cations and zinc competes with histamine for granule storage sites Conse quently Hi uptake is reduced and not enhanced in the presence of zinc in the suspension medium *In ummary* no e idence was found for a storage function of zinc in a heparin–zinc h stamine complex

Zinc has been found in rat peritoneal mast cells (Angyal and Archer 1968 Keller and Sorkin 1970) and in their granules (Amann 1962 Kerp 1963 Pihl and Gustafson 1967) Rats given Zn i p (Keller and Sorkin 1970) or i v (Fiedler *et al* 1970) incorporate the metal into their mast cells

Heparin and histamine form a complex *in vitro* by ionic binding between the sulphate ester groups of heparin and the amino groups of histamine (Fig 1 a) In the presence of zinc the amount of histamine that can be bound to heparin is doubled (Kerp 1963) presum ably due to the chelating action of zinc (Fig. 1 b) Since heparin zinc and histamine all occur in the mast cell granules it has been proposed that the histamine there is stored in a heparin zinc–histamine complex (Kerp 1963)

From extensive *in vitro* and *in vivo* studies on the storage properties of mast cell granules we have arrived at a different explanation for the histamine storage mechanism. The matrix of the mast cell granules consists mainly of a complex between heparin and a low molecular weight basic polypeptide In this complex the ester sulphate groups of heparin are linked to amino groups of the polypeptide and the granule matrix exhibits properties reminiscent of a weak cation exchange resin (Fig 1 c) Accordingly, *in vitro* the granules are able to take up and store both inorganic (*e g* sodium ions) and organic cations (*e g* histamine and sero-

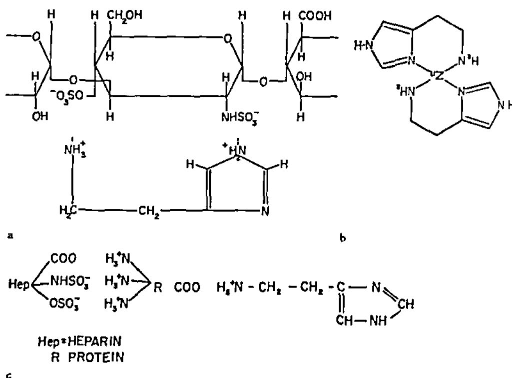


Fig 1 Assumed structure of a) *in vitro* heparin–histamine complex (from Uvnäs *et al* 1970) b) *in vitro* zinc histamine complex containing two molecules of histamine per metal atoms (from Green 1966) c) heparin–protein–histamine complex of mast cell granules at pH 7 (from Uvnäs *et al* 1970)

tonin) Presumably carboxyl groups in the granule complex constitute the ionic binding sites (Uvnäs Åborg and Bergendorff 1970) A theory that the release of histamine from mast cells is due to an ion exchange between the histamine in the granules and sodium ions in the extracellular fluid has been proposed and supported experimentally in various papers (for references see Uvnäs 1971)

In the mechanism of histamine storage as we imagine it to operate there is no obvious role for zinc However since there is no doubt that this metal is present in the mast cell granules we decided to reinvestigate its possible function in the granule amine store

Methods

Isolation of mast cells and their granules was performed according to Uvnäs Åborg and Bergendorff (1970) Male Sprague Dawley rats and a few Wistar rats (see Table 1) weighing 350–400 g were used

Studies on the uptake of Zn^{2+} by isolated granules were performed as described by Uvnäs *et al* (1970)

Zinc was determined either by activation analysis (AB Atomenergi Studsvik Nyköping, Sweden) or by atomic absorption spectrometry For the latter technique the zinc-containing samples (? ml of solution or suspension) were heated in 10 ml HNO_3 $HClO_4$ (5 1) The decomposition was continued until both the vapour fumes and the remaining solution were colourless. The heating was then carefully continued to dryness. 2 ml of aq dest. was added and after cooling the zinc-content was determined in an atomic absorption spectrophotometer (Zeiss) Duplicates showed variations below 10 %

TABLE I Zinc content of rat mast cells and granules isolated from such cells Each figure represents a single determination on material from 5 rats

nmol Zn/10⁶ cells		nmol Zn/mg gran	
Sprague Dawley rats	3 1[a]	Sprague Dawley rats	21 4[a]
	3 2[a]		13 8[a]
	3 1[a]		13 4[b]
	2.4[a]		13 3[b]
	4 1[b]		16 1[b]
			16 3[b]
			13 3[b]
Wistar rats	3 5[a]		
	6 1[a]		
	5.5		

[a] Atomic absorption spectrophotometry
[b] Activation analysis

Histamine was determined according to Shore Burkhalter and Cohn (1959)

Proteolytic activity was determined according to Lagunoff and Benditt (1963) on lysed cell material or on isolated granules in suspension (Uvnäs *et al* 1970) with casein as substrate and chymotrypsin as standard

Results

The zinc content in intact mast cells amounted to 3 2 (2 4–4 1 n = 5) nmol/10⁶ cells and in granules to 15 3 (13 0–21 4 n = 7) nmol/mg dry weight (Table I)

Since our zinc values were lower than those previously reported by other investigators (Angyal and Archer Pihl and Gustafson *l c*) we investigated the effect of the zinc content in the food Rats were fed for 3 weeks on diets free of zinc with a normal zinc content (18 6 p p m) or with a high zinc content (186 p p m) The diet used was obtained from the Nutrition unit Karolinska Institutet and based on the recommendations of the Association of Official Analytical Chemistry slightly modified only as to the source of nitrogen—all protein nitrogen was added as amino acids according to Wretlind (1972)[1]

The zinc content of the mast cells did not show any correlation to the zinc content of the diet However the zinc seemed to influence the histamine content and the chymase activity of the cells (Table II) The mast cells from rats on a diet with a high zinc content showed higher histamine content and chymase activity than the cells from rats on a zinc free diet

In order to observe possible seasonal variations the zinc determinations were performed at different times of the year between 1968 and 1974 No significant differences were found

Intracellular localization of zinc

After lysis of the mast cells in deionized water precipitation of the cell debris at 400 × *g* and of the granules at 3 000 *g* 20 of the zinc was found in the supernatant and 80 % in the granules and the granule-containing cell debris fraction (Table III) Histamine and chymase activity showed similar distributions When the lysed cell material was washed in

[1] The assistance of Gunnar Lev n in preparing the diets is gratefully acknowledged

TABLE II Effect of zinc content of the diet on zinc, histamine and chymase activity in mast cells and in their granules Each figure represents a single (zinc) or the mean of . (histamine and chymase activity) determinations on material from 5 rats.

Zinc content of diet	Zinc nmol per 10^6 cells	Zinc nmol per mg dried granules	Histamine nmol per 10^6 cells	Histamine nmol per mg dried granules	Chymase[a] µg per 10^6 cells	Chymase µg per mg dried granules
Zinc 186 mg/kg	3 7	9 2	202	618	73 2	381
Zinc 18 6 mg/kg	2.4	13 8	148	677	56 1	374
Zinc free	3 1	10 7	133	—	39 1	351

[a] Calculated as chymotrypsin.

0 9°$_o$ NaCl solution some differences in the affinities of the granule components (histamine zinc and chymase activity) to their storage sites were observed No histamine about 20 of the zinc but as much as 50°$_o$ of the chymase activity remained in the granules

Uptake of inc in mast cell granules in vitro

Mast cell granules have previously been demonstrated to take up and store cations *in vitro* (Uvnäs *et al* 1970) Zinc was found to be no exception The storage of zinc reached about the same maximum level as previously found for histamine and sodium *i e* about 1 000 neq per mg dried granule material (Fig. 2)

Influence of inc on granule histamine storage

The presence of zinc in the suspension medium reduced the uptake of histamine The reduction increased with rising zinc concentrations (Fig 3)

Incubation of the granules in the presence of 10 µeq/ml of zinc almost saturated the granule sites with zinc (Fig 2) Such zinc loaded mast cells showed a considerably reduced ability to take up histamine when subsequently incubated in the presence of the amine (Fig. 4)

TABLE III Distribution of zinc (expt 1–4) histamine and chymase activity (expt. 3–4) after lys s of m st cells in aq dest and subsequent incubation of isolated granules in isotonic NaCl
Each figure represents a single (zinc) or the mean of . (histamine and chymase activity) determinations on material from 5 rats.

	Zinc				Histamine		Chymase	
	1		3	4	3	4	3	4
Cell debris	30	38	31	34	49	17	35	31
Supernatant	30	14	3	1	6	6	15	13
154 mM NaCl	—		1	8	5	56	1	1
Granules	40	45	5	17	0	1	49	45

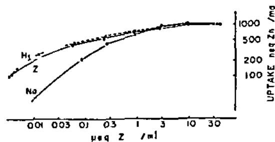


Fig   Uptake of sodium histamine and zinc by mast cell granules suspended in solutions containing various concentrations of these ions (curves for uptake of sodium and his tamine from Uvnäs *et al* 1970) ■—■ sodium ×—× histamine ●—● zinc

Discussion

In the mast cells zinc is mainly localized to the granules After lysis in water 80% of the zinc is recovered from the particulate fraction From the present experiments it is impossible to say how much of the 20% of zinc in the supernatant that emanates from granules or from other cellular constituents If ionically bound to the granule matrix part of the zinc will go into the water phase on lysis of the mast cells

As can be seen from Table III there is a marked difference between the affinities of histamine zinc and chymase activity for the granule matrix In agreement with previous reports the histamine is completely released when the granules are suspended in isotonic sodium chloride On the other hand about 20% of the zinc and an even larger proportion of the chymase activity is retained possibly indicating a more intimate association of these two constituents with the granule matrix than simple ionic binding

The proposal that histamine is stored in mast cell granules as a complex with heparin stems from the fact that *in vitro* the two substances form a rather stable complex by an ionic linkage between the ester sulphate groups of heparin and the amino groups of hista mine The complex binding requires a molar disaccharide–histamine ratio of one to one Quantitative data about the heparin and histamine content in granules are available only for rat peritoneal mast cells Such granules contain 30   heparin and 10   histamine cal culated on a dry weight basis Rat mast cell heparin is reported to be rich in ester sulphate groups According to Schiller and Dorfmann (1959) there are 3 13 ester sulphate groups per heparin disaccharide unit It can be calculated that if all these ester sulphate groups are

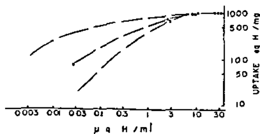


Fig 3 Influence of zinc in the suspension medium on the uptake of h stamine by mast cell granules *i vitro* ×—× No z nc in the suspension medium (from Uvnäs *et al* 1970) ●—● 0 3 meq zinc L in the suspension me dium ■—■ 3 μeq zinc/ml in the suspension medium

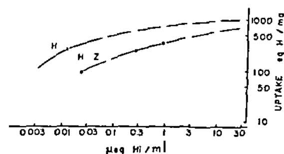


Fig 4 Reduced uptake of histamine by zinc loaded granules × — × Hi uptake in control ● — ● Hi uptake by zinc loaded granules

available for binding with histamine the heparin in the granules might just suffice to bind the histamine (Uvnas in press) However *in vitro* the heparin–histamine complex has its maximal stability at an acid pH (between 2–3) At pH 7 4 the affinity between histamine and heparin is reduced to about 10% of the maximum value since at this pH only one of the amino groups in histamine is ionized Furthermore salts reduce the formation of the complex which breaks down completely in 0 1 M sodium chloride Presumably therefore under the conditions likely to prevail in the mast cell granules the affinity between heparin and histamine is too low to allow sufficient complex formation to explain the histamine storage

In the presence of zinc the amount of histamine that can be bound to heparin *in vitro* is doubled (Fig 1 b) The discovery of zinc in the mast cells therefore seemed to increase the likelihood that histamine was stored in the granules in a complex with heparin The formation of such a zinc-containing complex requires at least 1 mol of zinc for each 2 mol of histamine We found only 2–4 nmol of zinc per 10 cells and maximally about 15 nmol of zinc/mg dried granule material The corresponding amounts of histamine were 150 and 680 nmol per 10^6 cells and mg dried granule material respectively In other words even if we assume that all the zinc found in the cells was localized to the granules the amount of zinc is far too low to explain the histamine storage as a zinc–heparin–histamine complex

Whatever the explanation for the differences in zinc values obtained by us and by others —they might be genetically or dietary determined—it does not alter the fact that the amounts of zinc present in our rat peritoneal mast cells—and in those of others—do not suffice to explain the histamine storage as a heparin–zinc–histamine complex There are also other arguments against the presence of such granule complex The formation of either a heparin zinc–histamine or a simple heparin–histamine complex requires available ester sulphate groups in the heparin By titration of the mast cell granules *in vitro* it can be shown that the granules lack such free sulphate groups (Uvnas Åborg and Bergendorff *l c*) indicating that in the granules the heparin ester sulphate groups are masked by an ionic linkage to amino groups of the basic polypeptides the other main constituents of the granule matrix

Due to the carboxyl groups in the heparin protein complex the granules show properties similar to a weak cation exchange resin The capacity of the granules to store histamine and sodium *in vitro* amounts to about 1 000 neq/mg dried granule material (Fig 2) a figure which agrees well with the titration values for weak acid groups presumably carboxyl groups in the granules The granules show an unselective uptake of organic as well as in

organic cations which compete for identical ionic binding sites (Bergendorff and Uvnas 1972) The granules should therefore also be able to bind zinc which would then compete with and not enhance the uptake of histamine The experiments illustrated in Fig 2–4 agree with this postulate The maximum amount of zinc taken up by the granules is approximately 1 000 neq/mg in other words the same value as previously found for histamine and sodium The presence of zinc in the granule suspension medium reduces the uptake of histamine the higher the zinc content the greater the inhibition Similarly loading the granules with zinc markedly reduces the uptake of histamine when the granules are subsequently suspended in a histamine-containing medium Evidently histamine has to compete with the zinc for common ionic sites All the observations cited are consistent with the assumption that zinc taken up by the granules *in vitro* is linked to the same storage sites in the granules as are sodium and histamine

The amounts of zinc found in our mast cells are about ten times lower than those previously reported by Angyal and Archer and by Pihl and Gustafson We have determined zinc with two techniques—activation analysis and atomic absorption spectrometry We have minimized the risks of seasonal and (possible) dietary variations by spreading our observations over several years Variations in the zinc content of the diet produced by lowering it to zero or increasing it to 10 times the normal values for about 3–4 weeks did not produce any significant changes in the zinc content of our mast cells At present we have no explanation to offer for the different zinc contents of our mast cells and of those of previous investigators

It has been suggested that in mast cells as well as in other zinc-containing cells zinc has functions other than those associated with storage The mast cell granules contain several enzymes which might require zinc for their activities Our findings of higher histamine content and higher chymase activity in mast cells from rats on a diet rich in zinc might reflect stimulating effects of zinc on enzymatic activities in the mast cells Proteins are known to chelate zinc (Klotz 1953) so it might function as a stabilizing factor in the protein part of the granule matrix Zinc has also been assumed to stabilize membranes (Chvapil 1973)

Zinc has also been claimed to play an essential role in the storage of other biogenic substances For example zinc occurs in the insulin-containing β-cells Since zinc forms a complex with insulin *in vitro* the conclusion has been drawn that insulin is stored in the β-cell granules in a complex with zinc (Maske 1960) However there are few quantitative data available about the insulin/zinc ratio and the amounts of zinc actually present in β-cells In some animal species the zinc content of the β-cells is minute (Maske 1960) The function of zinc in the storage of insulin and of other biogenic substances where the storage is supposed to be zinc dependent seems to require further evaluation

This investigation was supported by grants from Karolinska Institutet and the Swedish Medical Research Council (B74-14X 39 10C)

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Acta physiol scand 1975 93 409–414
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Distribution of an Adenohypophysial Constituent in the Body

I Whole-body Autoradiographical Studies in the Mouse

By

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Received 18 September 1974

Abstract

KIHLSTROM J E H HALL and E LAKOMAA *Distribution of an adenohypophysial constituent in the body I Whole body autoradiographical studies in the mouse* Acta physiol scand 1975 *93* 409–414

A peptide with a molecular weight of about 5 000 has pre iously been shown to affect the output of semen in frogs and probably also in mammals This sperm releasing substance is not part of any known gonadotrop c hormone The distribution of this substance has been in estigated using whole body autoradiography Radioactive material is incorporated into the epididymis the adenohypoph sis and probably also into the o ary

An extract from the adenohypophysis has been shown to induce the release of spermatozoa in frogs (Kihlstrom and Danninge 1970) The extract probably does not contain any known pituitary hormone (Lakomaa 1974 a, 1974 b) This activity has been demonstrated in extracts from pituitaries from frogs (Kihlstrom and Danninge 1970) perch rabbits cats cattle (Kihlstrom *et al* 1971) domestic fowls and humans (Lakomaa and Kihlstrom 1972) The pituitary extract has also been shown to increase the output of semen in rabbits guinea pigs and mice (Lakomaa and Kihlstrom 1972) An active constituent (molecular weight approximately 5 000) in the extract called sperm releasing substance as suggested by Lakomaa (1974 a) has been highly purified (Lakomaa 1974 b) and used in further studies (Lakomaa 1974 a) In the present work the distribution of the sperm releasing substance in the mouse is studied using the whole body autoradiographical technique

Material and Methods

An acetone powder prepared from bo ine adenohypophyses by Biofac A S (Copenhagen Denmark) was used when preparing the sperm releas ng substance according to Kihlström *et al* (1971) and Lakomaa (1974 b), excl d ng the final isoelectr c focusing. The sperm releasing substance can be lyoph l zed and stored for a cons derable t me

As the sperm releasing substance is a peptide probably containing at least one tyrosine residue (Lakomaa personal communication) it is possible to iodinate the substance with ^{125}I (Radiochemical Centre Amersham England) according to Greenwood et al (1963) 20 μg of the substance in 10 μl of distilled water and 10 or 50 μg Chloramine T in 10 μl sodium phosphate buffer pH 7 5 were exposed to 1 mCi Na ^{125}I (carrier free) in about 10 μl NaOH solution (pH 8–11) for 30 or 60 s respectively The reaction was interrupted by the addition of 24 or 1 0 μg respectively $Na_2S_2O_5$ in 50 μl 0 05 M phosphate buffer pH 7 5 The contents of the reaction vial were transferred to a Sephadex column (0 9 15 cm Sephadex G 25 or G 50 eluted with phosphate buffered saline containing 0 1 M EDTA and 0 002 NaN). The column was presaturated by the addition of 0 1 ml 20 Bovine Serum Albumin (BSA) Armour Fraction V Sigma and a subsequent addition of 1 mg of a crude preparation of the sperm releasing substance The reaction vial was washed with 0 1 ml 1 KI and this was also transferred to the column 10 μl of each eluate was diluted and the elution pattern was determined in a gamma counter type Autowell II The sperm releasing substance was eluted near the void volume and the free ^{125}I near the total volume The iodinated substance was stored at +4 C and was used within 10 days (often within ? days)

The mice used in the experiments were albino mice (NMRI) of about 2 months of age obtained from Anticimex Sollentuna Sweden

The autoradiographical procedure was similar to the procedure used by Ullberg (1954) About 10–30 μCi of the iodinated sperm releasing substance in 0 1–0 3 ml physiological saline was injected in a tail vein At different intervals after injection (15 s to 10 min) the mice were anesthetized with ether for about 30 s and rapidly frozen in a Dewar vessel containing hexane and dry ice (solid CO_2) (about −70 C). They were then immersed in a solution of carboxymethyl cellulose in water and the whole preparations were frozen in hexane – dry ice for about 15 min and then stored in a freezing room (−20 C) for 24 h or more The frozen blocks were sectioned in the freezing room with a microtome type Leitz to a thickness of 0–60 μm and dried at the same temperature X ray films (Industrex C Kodak) were then exposed to the sections for 3–8 weeks and the films were processed in Agfa Gevaert Developer G 150 and Agfa Geavert Fixer G 334

Observations

Radio-activity is found in many organs as listed below

Lung (*Fig 1*) No specific pattern of the distribution is found The activity never exceeds the activity of the blood

Liver (*Fig 1*) The activity never exceeds the activity of blood

Kidney (*Fig 1 and 4*) The activity of the kidneys is often dominating Most activity is found in the cortex

Ureter In the urine

Bladder In the urine

Pituitary (*Fig 2*) At about the same degree in both the anterior and the posterior part, though in some cases more activity in the anterior part

Secreting cells (*Fig 2 4 and 5*) Submaxillary gland epithelial cells of the nose mouth and small intestine mucosal cells in the walls of the stomach and thus also in the contents of the stomach

Thyroid (*Fig 2*) Always a great deal of activity

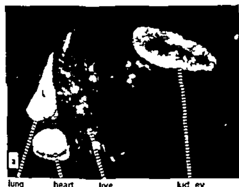
Epididymis (*Fig 3*) Especially in the caput epididymidis The activity seems to be localized to the walls of the tubuli

Testis (*Fig 3*) Very weak activity which seems also to be localized to the walls of the tubuli

Ovary (*Fig 4*) Follicles and or corpora lutea

Uterus Much weaker activity than in the ovary

Slight activity only is found in other male sexual organs (vas deferens seminal vesicle prostate coagulating gland)



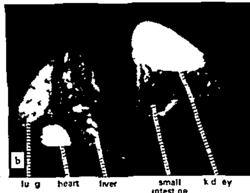


Fig. 1 a. Mal injected with 30 μCi of the iodinated sperm releasing substance Interval after injection 15 s, thickness .0 μm Exposure time .4 days Activity can be seen in the kidney lung liver and is very high in the blood.

Fig. 1 b Male injected with 30 μCi of the iodinated sperm releasing substance Interval after injection 1 min thickness 20 μm Exposure time 4 days Acti ity is found in the kidney lung li er small intestine stomach and blood Compare the differences between activity in the blood and in the kidney in this figure with that of Fig 1 a

Activity is at least to some extent found as soon as after 15 s in all organs mentioned except in the contents of the stomach where activity is only found after longer incubation times (Fig 5)

Discussion

The activity found in the different organs is not always the result of an incorporation of the iodinated sperm releasing substance It is probable that there is a great deal of free ^{125}I in the sections This can be due to both decomposition of the iodinated substance during storage and to a rapid decomposition in the animal during the incubation Since the distribution is studied after such short times a great deal of activity still remains in the circulating blood Therefore the activity found in organs rich in blood (Everett *et al* 1956) is not necessarily due to an incorporation of the sperm releasing substance

In the following list the probable cause of the activity found in the different organs is discussed



Fig. Male injected with 0 μCi of the odinated sperm releasing substance Interval after injection 15 s th ckness 40 μm Exposure time 14 days Act vity is found in the submaxillary gla d both parts of the hypophys s and the epithel ar cells of the nose The acti ity spots in the brain refer to blood essels

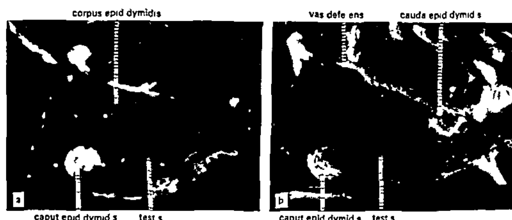


Fig 3 a Male injected with 20 μCi of the iodinated sperm releasing substance Interval after injection 1 min thickness 60 μm Exposure time 14 days High activity can be seen in the caput epididymidis while it is much lower in the other parts of the epididymis No activity is found in th testis

Fig 3 b Male injected with 30 μCi of the iodinated sperm releasing substance Interval after injection 3 min thickness 20 μm Exposure time ?4 days Activity is found primarily in the caput epididymidis but also in the other parts of the epididymis and in the vas deferens The very weak activity in the testis is located to the walls of the tubuli

Lung High blood content with remaining activity

Liver High blood content with remaining activity though also some metabolism of the iodinated sperm releasing substance (see below)

Kidney ureter and bladder Excretion of the iodinated sperm releasing substance and or free ¹³¹I

Pituitary Free ¹³¹I will accumulate in the neurohypophysis but not in the adenohypophysis (Jentzer 1953) In this study activity is found in the adenohypophysis indicating an incorporation of the constituent

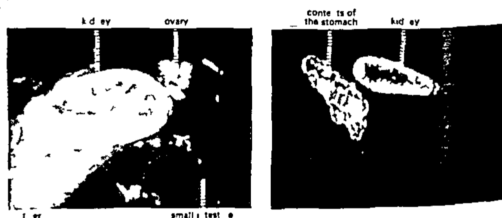


Fig 4 Female injected with 30 μCi of the iodinated sperm releasing substance Interval after i jec 15 s thickness ?0 μm. Exposure t me 4 days The foll cles and or the corpora lutea of the ov ry are incorporated w th the constituent Th pattern of the kidney is eas ly vi ible

Fig. 5 Male injected with 0 μCi of the iod nated sperm-releasing substance Interval after inject 10 m n thi kness 60 μm E posure time ays Acti ity is found almost only in the stomach and in t e ki ney

Secreting cells Accumulation of free ^{125}I (Ullberg and Ewaldsson 1964 Logothetopoulos 1956 a 1956 b)

Thyroid Accumulation of free ^{125}I

Epididymis Neither blood contamination nor incorporation of free ^{125}I seem to be the cause of the high activity found in the epididymis The blood content of the epididymis does not seem to be higher than that of the testis when comparing the colours of the two organs in the sections Radioiodine is not incorporated in the epididymis (Ullberg and Ewaldsson 1964) The picture of the incorporation of the sperm releasing substance in the epididymis and the testis is very similar to that of FSH (Rajaniemi 1971) From this similarity it can be concluded that this substance like FSH accumulates in the walls of the tubuli of the epididymis and testis The sperm releasing substance is not identical with FSH or with a fraction of FSH (Lakomaa 1974 a)

Ovary Free ^{125}I accumulates in the ovaries (Ullberg and Ewaldsson 1964) but so also does luteinizing hormone (Rajaniemi and Vanha Perttula 1972 Lee and Ryan 1971) It is unlikely that the sperm releasing substance is identical with LH since LH does not accumulate in the epididymis (Rajaniemi 1971) LH prepared in the same way as the sperm releasing hormone does not possess sperm releasing activity (Lakomaa 1974 a) It cannot be determined from these autoradiograms whether the accumulation in the ovary is due only to an incorporation of free ^{125}I

Uterus Probably blood contamination

The increasing amount of activity in the liver and in the kidneys with increasing intervals after injection is probably the result of the metabolism of the sperm releasing substance The increasing amount of metabolized substance will give free ^{125}I circulating in the blood leading to higher activity in the stomach after longer incubation times (Logothetopoulos 1956 a)

Thus the only organs where the activity seems to be due to an incorporation of the iodinated sperm releasing substance are the epididymis adenohypophysis and possibly the ovary The incorporation in the adenohypophysis indicates a mechanism of re uptake since the substance originates from this organ The incorporation in the walls of the caput epididymidis cannot at present be explained since the exact functions of the substance have not yet been investigated But it is not unexpected to find accumulation in this organ since the substance probably affects the output of semen also in mammals

A comparison between the distribution of the iodinated sperm releasing substance and that of LH and FSH can be made Both LH and FSH accumulate in the testis but the uptake is not visible in the autoradiograms after such short intervals as have been used in this study with the sperm releasing substance (Rajaniemi 1971) LH does not incorporate into the epididymis but FSH on the other hand does (Rajaniemi 1971) Thus the sperm releasing substance has a distribution pattern in the male sexual organs which differs from that of LH but it does not in this respect differ from that of FSH

This work has been supported by grants from the Helge Ax son Johnson Foundation and from the Swedish Natural Science Research Council to J n Erik Kihlström

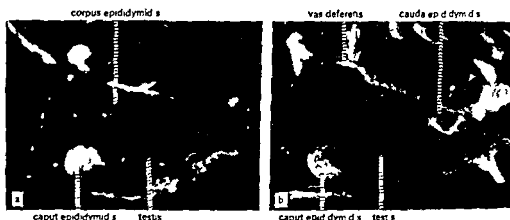


Fig. 3 a. Male injected with 20 μCi of the iodinated sperm releasing substance Interval after injection 1 min thickness 60 μm *Exposure time 14 days High* activity can be seen in the caput epididymid s, wh le it is much lower in the other parts of the epididymis No activity is found in th testis.

Fig. 3 b Male injected with 30 μCi of the iodinated sperm releasing substance Interval after injection 3 min thickness 0 μm Exposure *time* 4 *days* Activity is found primarily in the caput epididym dis, but also in the other parts of the epididymis and in the vas deferens The very weak activity in the test s is located to the walls of the tubuli

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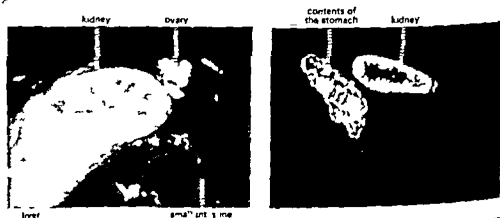


Fig 4 Female injected w th 30 μCi of the iodinated sperm releas ng substance Interval after i ject n 15 s, thickness 70 μm. Exposure time 4 days The foll cles and or the corpora lutea of the ovary are incorporated with the constituent The pattern of t e kidney is easily vi ble

Fig 5 Male injected with 70 μCi of t e iod nated sperm-releas ng substance Interval after i ject n 10 min thickness 60 μm E posure t me d s Act ity is found almost only i the stoma h and i the kidney

Acta physiol scand 1975 93 415–422

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The Diffusion Permeability to Water of the Rat Blood–Brain Barrier

By

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Received 23 September 1974

Abstract

BOLWIG T G and N A LASSEN *The diffusion permeability to water of the rat blood–brain barrier* Acta physiol scand 1975 93 415–422

The diffusion permeability to water of the rat blood–brain barrier (BBB) was studied Preliminary data obtained with the Oldendorf tissue uptake method (Oldendorf 1970) in seizure experiments suggested that the transfer from blood to brain of labelled water is diffusion limited More definite evidence of such a limitation was obtained using the single injection technique of Crone (1963) ^{14}C labelled sucrose was used as intravascular reference substance and tritium labelled water as test substance The non exchanging (transmitted) fraction I–E=T of labelled water during a single passage increased from 0 6 to 0 67 when the arterial carbon dioxide tension was changed from 15 to 85 mm Hg a change increasing the cerebral blood flow about sixfold This finding suggests that water does not pass the blood–brain barrier as freely as lipophilic gases

The concept of the blood–brain barrier (BBB) was originally based on the observation that the exchange of various dyes between blood and tissue does not occur nearly as easily in the brain as in other organs A quantitative approach to this phenomenon was made when Crone (1963) developed the indicator-dilution technique He demonstrated in dog experiments an extremely low permeability of brain capillaries to hydrophilic tracers such as inulin sucrose and fructose but in the case of D glucose a definite permeability was readily demonstrated with the characteristics of facilitated diffusion (Crone 1965) Using the same technique Yudilevich and De Rose (1971) found in dogs that sucrose and sodium cross the BBB so slowly that the permeability is too low to be measureable and Lassen *et al* (1971) demonstrated that also in man the blood–brain barrier has a very low permeability to small hydrophilic tracers (Lassen *et al* 1971) Crone (1965a) found however that low molecular weight aliphatic alcohols (*e g* ethanol) equilibrated with tissue in a single passage

Labelled water molecules are known to enter the brain rapidly (Yudilevich and De Rose 1971) In fact despite being hydrophilic water molecules are usually considered to belong to the same class of molecules as small lipophilic molecules such as gases for which the blood–brain barrier seems to offer a negligible resistance (Ter Pogossian *et al* 1970) On this

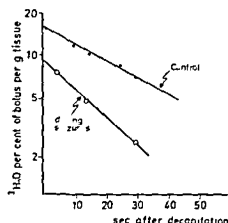


Fig 1 Semilogarithmic plot showing percentage uptake in brain of a 200 μl bolus of ^3H_2O injected into the common carotid artery against time after decapitation. Upper curve washout of ^3H_2O in control animals Lower curve washout of ^3H_2O during seizures

conceptual background tracer water has been used asa freely diffusible reference substance for the evaluation of blood–brain barrier transport of other substances (Oldendorf 1970 1971) and for measurement of cerebral blood flow (CBF) (Ter Pogossian *et al* 1970 Pappenheimer and Setchell 1973) However recent studies in monkeys by Raichle Eichling and Grubb (1974) showed a failure of labelled water to equilibrate completely with brain water during a single capillary transit this failure increasing progressively with increasing CBF Similarly evidence suggesting a diffusion limitation for water of the BBB in high flow situations has been found in rats by a tissue sampling technique after constant in fusion (Eklof *et al* 1974)

These findings prompted us to obtain quantitative data on the diffusion permeability of tracer water across the capillary wall in the rat brain with the aim of correcting the Oldendorf tissue uptake method for the incomplete water exchange and thus obtain quantitatively more valid data for the permeability of the blood brain barrier to various other hydrophilic molecules

Preliminary observations by Oldendorf s tissue uptake method

The impression that water does not equilibrate freely with brain water when cerebral blood flow is increased was gained as we studied the washout from brain of tritiated water injected as a slug into the common carotid artery The rats were decapitated at various intervals and the fractional uptake of the injected amount of tracer water was estimated Fig 1 shows a semilogarithmic plot of the retention of tracer water against time after decapitation. Upper curve represents the control state with artificially ventilated normocapnic animals, lower curve shows the situation during electrically induced epileptic seizures The lower curve was surprising for two reasons. First the extrapolated zero time value was considerably lower than in the controls and second the wash-out rate was not steeper During seizures we had expected a greater initial fractional uptake (zero line intercept) and a very steep outwash-curve having a slope about 3 times as steep not just twice as steep as the control curve This prediction was made because the state of seizure represents increase in CBF to about 300 of the control state (Plum, Posner and Troy 19(?)

Our tentative interpretation of these findings was that because of the increased CBF during seizure a substantial fraction of the injected water was transmitted through the cerebral microcirculation without traversing the capillary wall *i e* the extraction E of water had decreased

The more definitive observations by Crone s blood sampling method

Assuming that only brain drains to the superior saggital sinus then this sampling site may be used for testing the hypothesis that a sizeable fraction of the labelled water fails to equilibrate across the BBB at high blood flow rates in rats To obtain a quantitative assess ment of this we applied the double indicator dilution technique of Crone using sucrose as the non diffusible reference substance and tracer water as the test substance (Crone 1963)

General proced e

In Wistar rats weighing ca 300 g anesthesia was induced with halothane 4 delivered via a calibrated halothane vaporizer (Fluotec Keighley England) When the animals were unresponsive to external stimuli tracheotomy was performed and the animals were immobilized with D Tubocurarine 0 . mg/kg b wt intraperitoneally They were artificially ventilated with a rodent respirator (Braun Mehlsungen) An esthesia was maintained with a gas mixture containing nitrous oxide (N_2O) 70 and oxygen 30 The right femoral artery was surgically exposed and cannulated for anaerobic sampling before measurement of gas tens ons and pH The common carotid artery was likewise exposed before slug injection of the isotope m xture The incised tissues were infiltrated with lidocaine 1 The rectal temperature was meas ured with a mercury thermometer and kept close to 37 C by means of intermittant heating from a lamp bulb After e posing the calvarium a 1 2 mm burr hole was made with a dental drill in the center of the lambdoid process of the interparietal bone which is a reliable surface landmark overlying the region of the confluens sinuum The dura was left intact

Before carotid injection and venous sampling arterial pH pO_2 and pCO_2 were measured Only when the difference between 2 consecutive measurements with an interval of 10 min was less than 10 the animals were considered to be in a respiratory steady state Hypocapnia was obtained by passive hyper ventilation hypercapnia by adding 7 CO_2 to the inspired gas mixture Mean arterial blood pressure (MABP) was monitored via a pressure transducer connected with the femoral artery In all animals the PaO_2 was above 100 mm Hg and MABP above 100 mm Hg

R agent

The injectate cons sted of a mixture of 1 / Ci (^{14}C U) sucrose (Amersham England) and 1 μCi of ^{3}HOH (AEK Riso Denmark) in Ringer s solution buffered to a pH of 7 56 with 5 mM N 2 hydroxyethylpiper azine N ?-ethanesulfon c acid (HEPES) buffer The volume of the injectate was 00 μl

I ject n and s mpling

When steady state of respiration was obtained a slug inject on of the isotope mixture was delivered via a 27 gauge needle which is small enough not to occlude the vessel and thereby disturb the reestabl shment of flow after the inject on The injection lasted approximately 0 5 s S multaneously ith the injection manu l sampl ng in small preweighed glass tubes (Witrex pipettes) placed side by side between two lices of modelling wax was made from the surgically exposed confluens s nuum that had been op ned by a sharp needle just prior to the injection A new tube was placed in the burr hole e ery two second After reweigh ng each glass tube was emptied into a counting vial containing 0 5 ml of a mixture of soluene and d oxan

A aly cal te h ques

Arter l blood was analysed for pH and pCO using m croelectrodes operated at 37 C (Rad ometer Copen hagen)

The samples wh ch were all close to 0 / l were blanched w th 400 μl 35 H O 100 / l 1 M HCl and 15 ml scintillat on fluid (Instagel® P ckard) was added and count ng was made in a well type sc nt llation

counter Corrections were applied for quench and background as well as for channel spillover using the method of external standardisation

Calculations

The calculations are based on the equation derived by Crone (1963) who expressed the capillary permeability *i e* the amount of substance that passes unit area in unit time for unit concentration difference across the membrane Expressed in symbols this is

$$P = \frac{F}{S} \times \ln\left(\frac{1}{1-E}\right) \qquad (1)$$

where F is the tissue perfusion or blood flow and S the capillary surface area E is the extraction defined as the fractional transcapillary loss of test indicator in one passage through the capillary E may be expressed as $E = (C_a - C_v)\ C_a$ where C_a and C_v are the concentrations at the arterial and venous end of the capillary respectively The extraction value used in the calculations is the one corresponding to the peak of the curves Equation (1) may be rearranged to give the so-called Permeability Surface area product

$$PS = -F \quad \ln(1-E) \qquad ( )$$

PS has the same unit as flow and when multiplied with the fractional water content of blood it is expressed in ml water/100 g tissue × min

The flow values used in the calculations are those obtained by Eklöf *et al* (1973) using the Kety Schmidt method in rats of the same strain and under experimental conditions practically identical with those in the present study

Additional experiments

To further test the limitation of water as a freely diffusible tracer in brain we injected tritiated water with the highly diffusible ^{14}C nicotine (Amersham England) 1 µCi of each in a 00 µl volume as described abo e This was done in 4 consecutive expts

Results

Fig 2A B and C show 3 typical venous outflow curves and curves depicting the extraction (E) of labelled water The curves represent low normal and high flow situations The decreased flow in hypocapnia is well illustrated by the increased transit time of the intravascular tracer (Fig 2A) With increasing $PaCO_2$ and thereby increasing CBF the transmitted part of the labelled water (1–E) *i e* the fraction that remains within the cerebral vessels during a single capillary transit becomes larger (Fig 2B and C)

Table I gives the values of 1–E and of the calculated PS product The rates of flow (F) in ml/100 g min used in equation (1) were 64 (hypocapnia) 100 (normocapnia) and 465 (hypercapnia) (Eklof *et al* 1973) In spite of the decreased extraction of water during hypercapnia the PS product increased significantly compared to the values at normal and low $PaCO_2$ (*cf* Table I) showing that the experiments were conducted under partly flow limited and partly diffusion limited circumstances

Fig 3 shows typical venous outflow curves during normocapnia after injection of tritiated water and ^{14}C labelled nicotine In the 4 expts we found the peak value for nicotine to be 44 40 45 and 34% of the peak value for water

Discussion

The estimation of the extracted fraction of the test substance in the present study was made using the early part of the curves when the intravascular tracer concentration had reached

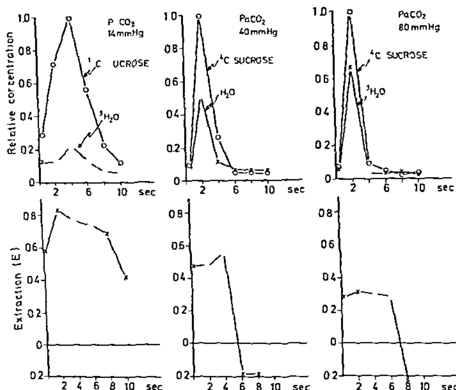


Fig 2 Upper curves show relative concentrations of C su rose and H O in sagittal sinus blood plotted against time after injection into the common carotid artery Lower curves show the extraction of H O calculated from the venous outflow curves Fig 2 A shows the situation in hypocapnia 2 B in normocapnia and 2 C in hypercapnia

its peak The higher the diffusibility of a test substance the larger the back-diffusion problem will be In the case of water which has a high diffusion permeability in brain capillaries a considerable degree of back-diffusion takes place as illustrated here by the early fall of the extraction curves to negative values (Fig 2)

Another question concerning the methodology is whether sagittal sinus blood may contain extracerebral venous admixture This question was studied by Murray and Plioplys

TABLE I Transm ssion (1–E) and permeabil ty surface area product (PS) of labelled water after slug in jection i to the carot d artery of the rat at var ous levels of PaCO Mean and range

Group	PaCO mm Hg	(1–E)	PS product ml water/100 g × mi t
Hypocapn a	15 0	0.26	71
(n = 5)	1..0–18 0	0 21–0 30	61–74
Normocapnia	38 5	0.53	53
(n = 5)	37 5–40 0	0 50–0 57	51–55
Hypercapnia	85 0	0 67	150
(n = 5)	80 0–90 0	0 65–0 68	140–15.

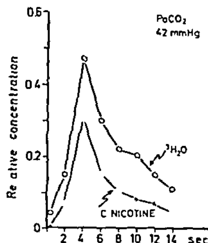


Fig. 3 Relative concentrations of ^3H_2O and ^{14}C nicotine in sagittal sinus blood plotted against time after injection into the common carotid artery

(1972) who in rat experiments simultaneously collected venous blood from the confluens of sinuses and the external jugular vein simultaneously after injection into the external and internal carotid artery respectively They found no significant extra-cerebral venous ad mixture to the blood from the sinus Thus sinus sagittalis seems to yield reliable information concerning the extraction of molecules by the brain

The finding that with rising CBF the non-extracted (transmitted) fraction of the injected bolus increases is quite in accordance with the results of Raichle *et al* in monkeys (1974) Using ^{15}O labelled water injected into the carotid artery and measuring via external gamma-detection they found that labelled water did not equilibrate freely This failure of exchange became more obvious as CBF increased above the normal level At normal CBF in the monkey (50 ml/100 g min) 90% of the injected bolus passed the BBB in a single transit which means almost complete equilibration

Raichle and co-workers calculated the PS product for water from their experiments with high CBF and found a PS value of approximately 138 ml/100 g min or 0 023 cm^3 s^{-1} g^{-1} Assuming an average capillary surface area (S) of the order of 200 cm^2/cm^3 and a water content of blood of approximately 0 81 ml per ml blood then the permeability P is 0 10 10^{-3} cm/s

The data from the present study allow a similar calculation of P for water in the rat If we use the PS value during normocapnia of 53 ml/100 g min or 0 009 cm^3 s^{-1} g^{-1} and the value for S indicated by Crone (1963) for cortical capillaries of 240 cm^2/cm^3 we get 0 04 10^{-3} cm/s This value is somewhat smaller than that of Raichle but not more than to be expected in view of the many approximations and uncertainties in the experiments

Our finding that normally only about 50 % of the labelled water equilibrated in the rat may well be explained by the high CBF in this small animal (100 ml 100 g min) Like Raichle *et al* (1974) we tested the possibility that our findings might be explained by the presence of arterio-venous shunts which might account for an apparent failure of complete water equilibration We injected a bolus consisting of ^{85}Sr labelled microspheres of 15 μm in diameter But neither at $PaCO_2$ 40 mm Hg nor at 80 mm Hg did the microspheres ap-

pear in the saggital sinus blood This means that arterio-venous anastomoses cannot explain our results

The above discussed findings demonstrating a diffusion limitation for water of the BBB are in contrast to those of Yudilevich and de Rose (1971) who concluded from their indicator-dilution studies that water is freely diffusible in the dog brain This discrepancy may be explained by the fact that the latter group used barbiturate which has been shown by many authors to reduce CBF in animal and man (Schmidt *et al* 1945 Himwich *et al* 1947 Gottstein 1961) or that the CBF for other reasons was lower than 50 ml/100 g × min

The Oldendorf technique rests on the assumption that there is no diffusion limitation for water across the brain capillary wall This is obviously not the case except when cerebral blood flow is quite low However with the present findings it seems possible to correct for the incomplete water exchange Oldendorf (1970) used the following calculation to estimate the uptake of a ^{14}C labelled substance in brain (Brain Uptake Index)

$$BUI = \frac{^{14}C \text{ tissue/ HOH tissue}}{C \text{ injectate/ HOH injectate}}$$

Table I gives the correction factors indicating a fractional uptake of water of 0 76 in hypocapnia at a $PaCO_2$ of 15 mm Hg 0 47 in normocapnia at a $PaCO_2$ of 39 mm Hg and 0 33 in hypercapnia at a $PaCO_2$ of 85 mm Hg It appears that the Brain Uptake Indices will be correspondingly lowered to yield better estimates of the extraction E

If a more permeable tracer than water could be found only insignificant corrections for loss of standard tracer would be necessary Not even nicotine though highly lipophilic behaves ideally as is seen in Fig 3 where a definite peak simultaneous with the water peak shows that during normocapnia a certain transmission of nicotine takes place If we assume a 50 % extraction of water (normocapnia) we would then find an extraction of nicotine about 70

Until a tracer more ideal than water can be applied quantitative data obtained with the Oldendorf technique should be interpreted with caution unless as in the present study constancy of CBF at well-defined levels during the experiments is ascertained

Concerning Oldendorf's own data they have been obtained under experimental conditions that did not differ from animal to animal and the data concerning blood to brain transfer of a number of substances obtained in Oldendorf's laboratory therefore give the correct hierarchy of permeabilities

The work was supported by grants from Det Lægevidenskabelige Forskningsråd and from Fonde t l Lægevid nskabens fremme

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Acta physiol scand 1975 93 423–432
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The Relationship between Arterial P_{O_2} and Cerebral Blood Flow in Hypoxic Hypoxia

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Received 26 September 1974

Abstract

BORGSTROM L H JÓHANNSSON and B K SIESJÖ *The relationship between arterial* P_{O_2} *and cerebral blood flow in hypoxic hypoxia* Acta physiol scand 1975 93 423–432

The relationship between arterial oxygen tension (Pa_{O_2}) and cerebral blood flow (CBF) in hypoxic hypoxia was studied in artificially ventilated and normocapnic rats. Changes in CBF were evaluated from arteriovenous differences in oxygen content after 2 5 15 and 30 min exposure to Pa_{O_2} 85 75 55 45 35 and 25 mm Hg In separate experiments the Pa_{O_2} was decreased to 25 mm Hg for 1 2, 5 15 and 30 min in animals in which Pa_{CO_2} was allowed to fall by 5–10 mm Hg. There was a small gradual increase in CBF when Pa_{O_2} was lowered in steps from 130 to 55 mm Hg, and a more pronounced increase at P_{O_2} values below 50 mm Hg At Pa_{O_2} 25 mm Hg CBF increased to values of 500 % of normal Significant increases in CBF were recorded at Pa_{O_2} values of 85 and 75 mm Hg in spite of the fact that previous studies have failed to record an elevated tissue lactate content at these P_{O_2} levels and in spite of an unchanged cerebral venous P_{O_2} When the Pa_{O_2} was reduced to 25 mm Hg CBF increased markedly already at 1 and 2 min and this increase in CBF occurred even if Pa_{CO_2} was allowed to fall by 5–10 mm Hg. Previous results have shown that in such short periods enough lactic acid is not formed to induce a net tissue acidosis The results thus give no support to the hypothesis that cerebral hyperemia in hypoxia is coupled to accumulation of lactic acid in the tissue

It has been widely assumed that the increase in cerebral blood flow (CBF) which occurs under conditions of hypoxic hypoxia is elicited by an insufficient oxygen supply to the brain (see *e g* Opitz and Schneider 1950) In view of the fact that the cerebral resistance vessels are exquisitely sensitive to changes in arterial CO_2 tension it has been proposed that an increased H^+ activity in the cerebral extracellular fluids is responsible for the vasodilatation both in hypercapnia and in hypoxia (see Lassen 1968 Betz 1971) Two facts support this assumption *Firstly* several studies have shown that the cerebral vasodilatation in hypoxia is a threshold phenomenon that occurs first when the Pa_{O_2} is reduced below about 50 mm Hg (Noell and Schneider 1942, McDowall 1966 Kogure *et al* 1970) and since brain lactate levels are not elevated until Pa_{O_2} is reduced to this level (Siesjo and Nilsson 1971) a coupling between lactic acidosis and increased CBF has seemed plausible Secondly Kogure *et al* (1970) failed to record an increase in CBF in hypoglycemic dogs exposed t

6% O_2 and reasoned that the hyperemic response was abolished due to lack of substrate for cerebral glycolysis

The metabolic theory of CBF regulation in hypoxia requires that there is a quantitative relationship between tissue lactate levels and degree of hyperemia at least if the arterial P_{CO_2} and mean arterial blood pressure are held reasonably constant Since information on tissue lactate levels in the rat is available both for semi-steady state hypoxia at Pa_{O_2} levels of 150-20 mm Hg (Siesjo and Nilsson 1971) and for non-steady state hypoxia at Pa_{O_2} 25 mm Hg (Norberg and Siesjo 1974) we decided to measure CBF under comparable experimental conditions We have recently demonstrated that the cerebral metabolic rate for oxygen (CMR_{O_2}) remains unchanged even if Pa_{O_2} is reduced to 22-25 mm Hg (Jóhannsson and Siesjo 1973 1974) In the present study changes in CBF in response to hypoxia were therefore evaluated from the arteriovenous differences in oxygen content (AVD_{O_2})

It will be shown that under semi-steady state conditions there is an increase in CBF at degrees of hypoxia that do not lead to a measurable elevation of tissue lactate content. Furthermore under non-steady state conditions CBF increases markedly before there is a significant degree of cerebral lactic acidosis The results thus lend no support to the hypothesis of a metabolic regulation of CBF in hypoxia A preliminary account of the findings was given at the VIIIth Benzon Symposium in Copenhagen (Siesjo *et al* 1974) At that time we were unaware of the work of Ponte and Purves (1974) who showed that the CBF response to hypoxia in the baboon depends on impulses from the carotid body The present results are in agreement with the conclusions of Ponte and Purves (see Discussion)

Methods

The experiments were performed on male Wistar rats (3 0–410 g) that were given rat pellets and tap water *ad lib tum* Anesthesia was induced with ~3 halothane the animals were then tracheotomized immobilized and artificially ventilated with a mixture of 70 N_2O and 30 O_2 so as to give an arterial CO_2 tension of about 35 mm Hg. Both femoral arteries were cannulated for blood pressure recording and sampling of arterial blood and one femoral vein for infusion of fresh donor blood The posterior part of the superior sagittal sinus was exposed for sampling of cerebral venous blood The body temperature was measured in the rectum and kept close to 37 C by means of intermittent heating.

After the completion of the operative procedures a steady state period of 30 min was allowed At the end of this period blood pressure and body temperature were noted and hemoglobin concentration arterial P_{CO_2} and pH as well as arterial and cerebral venous P_{O_2} and total oxygen content (T_{O_2}) were measured The oxygen content of the inspired air was then reduced so as to give arterial P_{O_2} levels of about 85 75 55 45 35 and 25 mm Hg. During the hypoxic period the N_2O concentration was kept at about 70 by means of admixture of nitrogen gas At marked degrees of hypoxia a fall in Pa_{CO_2} was avoided by addition of carbon dioxide to the gas mixture delivered to the respirator After 5 15 and 30 min arterial and cerebral venous samples were simultaneously collected for measurements of P_{O_2} and T_{O_2} At each occasion blood pressure and body temperature were noted and Pa_{CO_2} measured Arterial pH and hemoglobin concentration were determined again at 30 min During the whole experimental period a slow infusion of donor blood was given to replace blood losses thereby maintaining a reasonably constant blood pressure A control group was obtained by maintaining ventilation on the original gas mixture

In order to allow comparisons with a recently published report of changes in tissue lactate concentration in hypoxic hypoxia (Norberg and Siesjö 1975) a separate group of animals were exposed to hypoxia (Pa_{O_2} about 25 mm Hg) without addition of carbon dioxide to the gas mixture In these animals the Pa_{CO_2} fell by 5–10 mm Hg (see below) Blood samples were obtained at 0 1 2. 5 15 and 30 min for AVD_{O_2} measurements.

Since AVD_O was measured in all animals prior to the induction of hypoxia each animal served as its own control and the changes in CBF were calculated (in per cent) as $[AVD_O]_n$ $([AVD_O]_h)^{-1}$ 100 where n and h refer to normoxia and hypoxia respectively There were 4–5 animals in each group However since the previous study of CBF and CMR_O (Johannsson and Siesjö 1974) involved a similar experimental protocol, the results of that study were included (15 and 25 min of hypoxia) provided that the Pa_O values obtained were within ±3 mm Hg of the P_O levels chosen for this study At Pa_{O_2} 25 mm Hg a satisfactory mean arterial blood pressure (≥100 mm Hg) could not be upheld for 30 min. The values given for 25 min of hypoxia at Pa_O 25 mm Hg were therefore taken from the previous study

P_{O_2} P_{CO_2} and pH were measured with microelectrodes with due correction for body temperature The oxygen content of arterial and cerebral venous blood were measured on 25 μl samples using the method of Fabel and Lübbers (1964 see Borgström *et al* 1974)

Statistical differences were evaluated with the Student s t test.

Results

I *Changes in CBF at constant* Pa_{CO}

In several previous studies of CBF in hypoxia accidental changes in Pa_{CO} or mean arterial blood pressure have complicated the interpretation of changes in flow (see discussion) Table I shows that Pa_{CO_2} could be held constant at about 35 mm Hg in all experimental groups Under the present experimental conditions (normocapnia nitrous oxide anesthesia immobilization with tubocurarine chloride) the induction of hypoxia was accompanied by a small (10–20 mm Hg) increase in blood pressure that lasted less than 1 min As is shown in Fig 1 the pressure then returned to normal or subnormal values At Pa_O 45 mm Hg, or lower blood pressure fell by 20–25 mm Hg over the 15–30 min period The data thus exclude that increases in Pa_{CO} or mean arterial blood pressure could have contributed to the recorded increases in CBF

In all groups body temperature was kept close to 37 C At any one Pa_O level the largest difference in mean body temperature was 0 7 C and since any differences observed were non-systematic temperature differences cannot have influenced the results The lowest blood hemoglobin content measured in any animal was 15 g (100 ml)

Fig 2 defines the degree of hypoxia in terms of Pa_O and arterial T_O In this figure the 1 min values (see below) were included for comparison In general Pa_O remained stable throughout the hypoxic period while arterial T_O fell gradually in all hypoxic group with a Pa_O of 55 mm Hg or lower This fall was especially marked at Pa_O 35 and 25 mm Hg where arterial T_O was reduced to half between 1 and 30 min The gradual desaturation was obviously related to a fall in plasma pH that was proportional both to the degree of hypoxia and to the time of hypoxic exposure (*cf* Siesjo and Nilsson 1971 Norberg and Siesjo 1975)

The relationship between arterial and cerebral venous P_{O_2} is given in Fig. 3 There was no decrease in Pv_O until Pa_O was reduced below 70 mm Hg. At Pa_O 35 and 25 mm Hg Pv_O decreased with time of exposure Previous values obtained after 30 min of exposure to Pa_O 20–25 mm Hg have given Pv_O values close to 10 mm Hg (MacMillan and Siesjo 1972)

The percentage changes in CBF as calculated from AVD_{O_2} are given in Fig 4 In general CBF increased progressively with the decrease in Pa_{O_2} Two findings should be emphasized Firstly at any Pa_O level the increase in CBF was maximal at 2 min Secondly there was an increase in CBF when Pa_O was reduced from 130 to 85 and to 75 mm Hg, and at Pa_O

TABLE 1. Arterial P_{CO_2}, in mm Hg, at different periods and levels of hypoxia. Values are arithmetic means ±S.E.

P_O level mm Hg	Period of hypoxia (min)				
	0	2	5	15	30
Control	36.3±0.6	35.2±0.8	35.8±0.4	36.3±0.7	36.7±1.0
85	37.0±0.5	35.8±0.6	36.3±0.8	36.5±0.7	36.5±0.6
75	35.8±1.0	35.6±0.8	36.3±0.4	37.0±0.6	37.3±0.9
55	34.8±0.8	35.2±0.3	36.2±0.4	36.5±0.9	36.9±0.7
45	36.6±0.7	37.4±1.2	38.3±1.0	37.4±0.8	37.2±1.9
35	35.0±1.1	35.5±0.6	35.6±0.6	35.6±1.1	34.9±1.0
25	35.5±1.1	37.2±0.9	36.7±0.9	34.5±0.8	— —

55 mm Hg CBF increased to 140–160% of normal. Since there were no significant differences between CBF values obtained after 2, 5, 15 and 30 min at any one Pa_O, level, the CBF figures were pooled. At Pa_O, 85 mm Hg the values for 2, 5, 15 and 30 min gave a mean CBF of 111% (±S.E. of 2%, n = 20). When this value is compared to the 2–30 min values for the control group (CBF 92±3%, n = 16) a statistically significant difference is obtained ($p < 0.001$).

Fig. 5 shows the relationship between Pa_{O_2} and percentage change in CBF for pooled values. The maximal increases obtained (at Pa_O, 25 mm Hg) are in excellent agreement with the CBF values obtained using the Kety and Schmidt technique (1948) for measuring CBF (Jóhannsson and Siesjö 1974).

II. *Changes in CBF at reduced Pa_{CO_2} values*

In these experiments the Pa_O, was reduced to about 25 mm Hg with no addition of CO, to the gas mixture. Since the protocol for these experiments was identical to that used in the previous study of tissue metabolites (see Norberg and Siesjö 1975) Pa_{CO}, was measured at 15 and 30 min only. At these times Pa_{CO}, was reduced by 7±1 and 11±1 mm Hg, respectively, quite in agreement with the previous study. Fig. 6 illustrates the Pa_O, and CBF values obtained. The data indicate that the development of hypocapnia somewhat curtails the increase in CBF during hypoxia (*cf.* Fig. 4). However, the results show that CBF increases to maximal values (about 400% of normal) during the first 2 min of hypoxia (see Discussion).

Discussion

There have been several previous studies of CBF as a function of Pa_O, in hypoxic hypoxia. Some of these have utilized CBF methods that are usually considered quantitative. Thus, McDowall (1966), estimating CBF of the exposed cat cortex from the rate of clearance of Kr, reported that CBF increased first when the Pa_O, was reduced below about 50 mm Hg, and obtained maximal CBF values of 140% of normal at Pa_O, values of about 30 mm Hg. Other workers using semi-quantitative or qualitative estimates of CBF have reported similar results. Thus, using a venous outflow technique in the dog, Kogure *et al.* (1970)

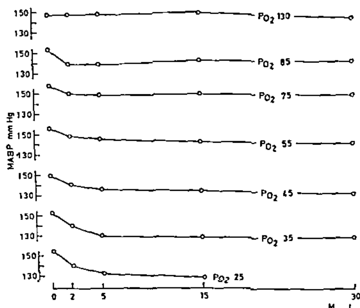


Fig 1 Mean arterial blood pressure (MABP) in mm Hg as a function of time for different levels of hypoxic hypoxia Each po nt represents the mean of 4 values

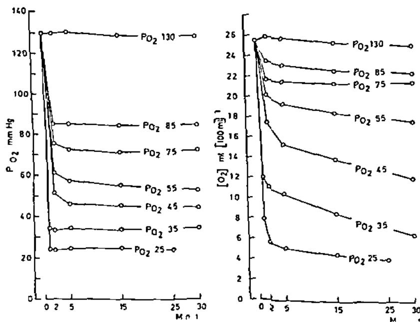


Fig. 2. Arterial oxygen tens on (PaO_2) and total arterial oxygen content ([O]) as functions of t me for d fferent le els of hypox c hypo a As indicated in the figure the Po levels chosen were 130 85 75 55 45 35 and 5 mm Hg respect vely Each point represents the mean of 3–4 val es.

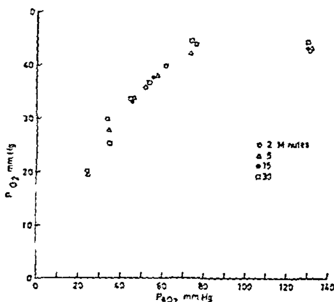


Fig. 3 Cerebral venous oxygen tension ($P\bar{v}O_2$) as a function of arterial oxygen tension (PaO_2) in hypoxia of 2–30 min duration. Each point represents the mean of 3–4 values

arrived at the same conclusion *i e* that CBF increases first when Pa_{O_2} is reduced to about 50 mm Hg. In the experiments of Kogure *et al* the CBF increased to maximal values of about 250% of normal

In the experiments of McDowall relatively few measurements were performed at Pa_{O_2} values exceeding 50 mm Hg, and in those of Kogure *et al* (1970) only 10 and 6 ... O_2 were tested. Since the results do not conclusively demonstrate a threshold at Pa_{O_2} 50 it seems warranted to scrutinize also other studies. It is now well established that the CMR_{O_2} remains unchanged when Pa_{O_2} is reduced to 40–35 mm Hg in man (Kety and Schmidt 1948, Cohen *et al* 1967) and to 20–25 mm Hg in the rat (Johannsson and Siesjo 1974). This makes it possible to estimate changes in CBF from the arteriovenous differences in oxygen content. The AVD_{O_2} method is superior in sensitivity to most other techniques, it does not require extensive surgery or exposure of brain tissue and it allows each animal to serve as its own control. Using this method Courtice (1941) and Noell and Schneider (1942) reported that there was an increase in CBF at a Pa_{O_2} of about 45 mm Hg. Although these results are in accordance with those of McDowall (1966) and Kogure *et al* (1970) spontaneous respiration was employed and any tendency of vasodilation at Pa_{O_2} values higher than 50 mm Hg could have been nullified by the accompanying hypocapnia. In addition the large pressure effects obtained in the preparation of Noell and Schneider (1942) complicate evaluation of the increases in CBF

The present experiments offer some advantages compared to previous studies. Firstly the experiments were performed in light (N_2O) anesthesia which has minimal effects on CMR_{O_2}. Secondly animals were studied after identical periods of hypoxia at a wide range of O_2 tensions. Thirdly the Pa_{CO_2} was held constant. Finally there was no increase in blood pressure at any experimental time. The data clearly establish that there is an increase in CBF at Pa_{O_2} values exceeding 50 mm Hg. In fact the data suggest that there is a continuous

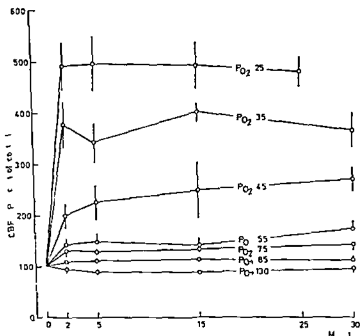
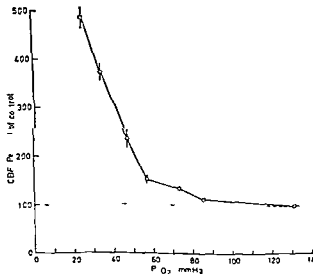


Fig. 4 Changes in cerebral blood flow (CBF) as calculated from the arteriovenous differences in oxygen content in hypox a of 2–30 min duration The gross Po levels are indicated in the figure The values are means ± S E. for groups of 3–5 an mals



F g. 5 Cerebral blood flow (CBF) calculated from the arteriovenous d fferences for oxygen content, as a function of arterial oxygen tension (PaO) Each po nt repre sents the mean of alues for hypoxia of 5 15 and 30 min duration (n = 16 n all cases except for PaO = 85 where n = 20) Ver tical bars ind ate ± S E.

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Acta physiol scand 1975 93 433–441

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Incorporation of Leucine into Human Skeletal Muscle Proteins

A Study of Tissue Amino Acid Pools and their Role in Protein Biosynthesis

By

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Received 26 June 1974

Abstract

Lundholm K and T Scherstén *Incorporation of leucine into human skeletal muscle proteins A study of tissue amino acid pools and their role in protein biosynthesis* Acta physiol scand 1975 93 433–441

The *in vitro* incorporation of labelled leucine into human skeletal muscle proteins was studied with the aim to elucidate the relationship between the amino acid tissue pools and protein biosynthesis. The distribution volumes of leucine and cycloleucine in skeletal muscle tissue were similar but the equilibration time was shorter for leucine than for cycloleucine The cellular uptake of leucine and cycloleucine was competitively inhibited by increased concentration of amino acids in the medium indicating an active transport. Optimal stimulation for incorporation of leucine into proteins was obtained at an amino acid concentration in the medium corresponding to 10 times that of normal human plasma The incorporation of ^{14}C leucine into skeletal muscle proteins was linear before the total pool of free intracellular ^{14}C leucine had reached an equilibrium The regression coefficients for the semilogarithmic plots of the labelling rate of proteins with ^{14}C leucine and the incorporation rate of leucine calculated from the specific activity in the medium versus the amino acid concentration in the medium were different in the same experiment indicating a reutilization of amino acids released at protein degradation The results are compatible with the hypothesis that the proteolytically released amino acids have a competitive advantage for incorporation as compared with extra and intracellular free amino acids. It is concluded that the amino acid pool which is in the immediate continuity with the protein biosynthesis sites equilibrates rapidly with the extracellular amino acid pool.

Several studies of the amino acid pools and their role in protein biosynthesis in various mammalian tissues bacteria and yeast have been presented (Kipnis Reiss and Helmreich 1961 Rosenberg Bergman and Segal 1963 Guidotti Ragnotti and Rossi 1964 Gan and Jeffay 1967 Roscoe Eaton and Choy 1968 Hider Fern and London 1971 Morgan *et al* 1971 Mortimore Woodside and Henry 1972, van Venrooy *et al* 1972, Fern and Garlick 1973) Results from the *in vitro* studies indicate that the isotopic label in the presumed precursor pool equilibrates more rapidly with the extracellular amino acids than with the total intracellular pool of free amino acids In organ perfusion studies Morgan *et al* (1971) have clearly shown that the intracellular pools of glycine and lysine are on the pathway of protein synthesis thus indicating an intracellular location of the immediate precursor pool

for protein biosynthesis. In this context it is important to emphasize that the operationally defined total intracellular amino acid pool may comprise the sum of different functionally or morphologically compartmented intracellular pools of amino acids (Randle and Smith 1958, Norman *et al.* 1959, Allfrey *et al.* 1961, Cowie 1962, Nukada 1965, Mortimore 1972). It seems obvious, at least from these *in vitro* studies, that the intracellular pool is not functionally homogeneous with respect to protein synthesis.

So far no method for determination of the metabolism of proteins and the turnover rate of proteins in human skeletal muscle tissue has been presented. Furthermore, it appears very difficult to design an *in vivo* method for such determinations. Recently we described a human skeletal muscle preparation suitable for metabolic studies *in vitro* (Holm and Scherstén 1972, Lundholm *et al.* 1974). With this preparation it was possible to obtain zero order kinetics and an optimal stimulation for incorporation of leucine into muscle proteins (Lundholm *et al.* 1974). This model seems to create a possibility for at least a qualitative analysis of protein metabolism in human skeletal muscles. One prerequisite for obtaining a more quantitative measure of the biosynthesis and the turnover rate of proteins seems to be techniques for determination of the degree of amino acid reutilization from protein degradation.

The present investigation was designed to study the relationship between the different pools of radioactive amino acids and incorporation of leucine *in vitro* in human skeletal muscles.

Material and Methods

Reagents and isotopes

Inulin-carboxyl ^{14}C 1–3 mCi/g, D-sorbitol U-^{14}C 150–250 mCi/mmol, L-leucine U-^{14}C 250 mCi/mmol, L-leucine-4,5-^{3}H 5 mCi/mmol, 1-amino-cyclopentane-1-carboxylic acid carboxyl-^{14}C 10 mCi/mmol, α-amino-isobutyric acid (AIB)-1-^{14}C 1.5 mCi/mmol, $^{3}H_2O$ 100 mCi/g were obtained from New England Nuclear Chemicals (West Germany).

A sterile amino acid solution consisting of 19 L-amino acids with a concentration equivalent to 100 times that of human plasma was kindly supplied by AB Astra, Södertälje, Sweden.

Muscle tissue

Biopsies from the rectus abdominal muscle were taken at operation of patients with uncomplicated gall stone disease or peptic ulcer disease. The patients were fasted 12 h before the operation and were premedicated 1 h before the general anesthesia by means of diazepam and atropine. The anesthesia consisted of hexobarbital (Evipan®), oxygen and nitrous oxide. The biopsies were taken as the first procedure during the operation and without unnecessarily touching the muscle.

Preparation of muscle tissue, incubation and preparation of protein

The biopsies were prepared and incubated as described in detail earlier (Lundholm *et al.* 1974). Muscle fibers were teased away from the specimen and collected in incubation vessels. In each vessel 150–200 mg tissue was incubated in a medium consisting of KRB buffer, pH 7.4, radioactive compound and a mixture of 19-L amino acids in concentrations expressed as multiples of the human plasma concentration.

The incubations were performed in a metabolic shaker at 37 °C and constant agitation (80 cycles/min) for various periods of time (7 min – h). Before incubation the tubes were gassed for 1 min with O_2: CO_2 (95:5) and then sealed with tightly fitted rubber stoppers. After incubation the muscle tissue was blotted on a filter paper in a standardized manner, or washed in ml ice-chilled KRB-buffer for 15 min and then homogenized in an Ultra Turrax homogenizer (Janke and Kunkel, West Germany).

Protein preparation was performed according to the earlier description (Lundholm *et al.* 1974).

Determination of radioactivity

These determinations were performed after careful standardization of the handling of the tissue and solutions. After incubation, the tissue was either blotted on a filter paper to determine total radioactivity or washed in an ice-chilled buffer solution to determine the intracellular radioactivity (Guidotti Ragnotti and Rossi 1964) in accordance with our previous description (Lundholm *et al* 1974) Tritiated water was used to determine the distribution volume of diffusible water in the tissue

The radioactivity in the water solutions was determined in Instagel® (Packard Instrument Comp USA) and non aqueous samples in PPO POPOP toluene Correction for quenching was performed by the external standard method The radioactivity was given per protein weight, determined according to Lowry (1951) using human plasma albumin as a standard. The efficiency of the radioactivity counting in protein was tritium 17–0 percent ^{14}C 78–83 percent, and in the TCA supernatant tritium 22–23 percent ^{14}C 74–78 percent.

Results

Distribution volumes of the tissue (Fig 1)

^{1} C-cycloleucine was distributed at a slower rate than ^{14}C leucine but gave approximately the same distribution volume These isotopes were obviously accumulated against a concentration gradient since they showed larger distribution volumes than diffusible water ^{14}C–D sorbitol seemed to enter the intracellular space and showed a different distribution volume than for instance ^{1}C 3 O-methyl glucose and ^{14}C inulin (Lundholm *et al* 1974) The cellular uptake of ^{14}C-cycloleucine and ^{14}C AIB was possible to inhibit competitively

Intracellular distribution volumes

Amino acids remaining in the tissue after equilibration at +37 C and subsequent washout in ice-chilled buffer solution were defined as being of intracellular distribution (Lundholm *et al* 1974) Table I showes the decrease in the intracellular distribution volume of ^{14}C cycloleucine ^{1} C AIB and H leucine with increasing amino acid concentration in the medium The ratios between intracellular and extracellular distribution volumes were almost the same for all three labelled compounds The extracellular distribution volume was calculated from the total tissue distribution volume minus the intracellular distribution volume

Incorporation rate of C leucine into proteins and into the tissue intracellular pool

The incorporation rate of leucine into tissue proteins was calculated from the specific activity in the medium Optimal stimulation was obtained at a medium concentration of amino acids corresponding to 10 times that of human plasma In some experiments a tendency to a biphasic substrate stimulation curve was seen with an initial saturation level at an amino acid concentration corresponding to 4 times that of plasma ^{14}C leucine was linearly incorporated into TCA precipitable protein during incubation for 7 min to 2 h (Fig 2) Table II shows the stimulation of the incorporation rate of leucine into proteins by a higher concentration of amino acids in the medium and that ouabain as well as monoiodoacetate inhibited the incorporation rate of leucine Puromycin extinguished the incorporation almost completely

The uptake rate of leucine into the intracellular pool of free amino acids increased almost linearly with increasing concentrations of amino acids in the medium (from 1 to 16 times the concentration of normal plasma)

TABLE I Distribution of ^{14}C-cycloleucine ^{14}C-α aminoisobutyric acid and ^{3}H leucine in human skeletal muscle preparation Mean ± S.E.

Labelled compound	Medium amino acid concentration mmol l	Intracellular space (ml × g prot^{-1})	Extracellular space (ml × g prot^{-1})	Intracellular space / Extracellular space
^{14}C-cycloleucine	Normal plasma level	3 7 ± 0 2 (8)	2 8 ± 0 4 (3)	1 3.
	10 times plasma levels	2 9 ± 0 2 (9)	—	—
^{14}C AIB	Normal plasma level	3.2 ± 0 4 (3)	2 4 ± 0 3 (3)	1 33
	4 times plasma level	3 1 ± 0 2 (8)	—	—
	10 times plasma level	2 5 ± 0 2 (8)	—	—
^{3}H leucine	Normal plasma level	3 8 ± 0 1 (8)	2 9 ± 0 2 (3)	1 31
	10 times plasma level	3 6 ± 0 2 (8)*	—	

P < 0 01 Wilcoxon's test for pair differences (Siegel 1956)

In Fig 3 the labelling of proteins with ^{14}C leucine and the incorporation rate of leucine into proteins as calculated from the specific activity in the medium are plotted versus the amino acid concentration in the medium in a semilogarithmic scale The regression coefficients of these lines were different (k = 6 2 and 5 4 respectively) giving a ratio between them of 1 15

Interrelationship between the ^{14}C leucine tissue pools and the incorporation rate of leucine into proteins

The incorporation of ^{14}C leucine into proteins was linear from 7 min and on i e before the total pool of free intracellular ^{14}C leucine had equilibrated (Fig 2) There was a tendency to a lag phase during the first 7 min probably reflecting the distribution time of labelled compound between the incubation medium and the immediate precursor pool for protein synthesis

To further elucidate whether extracellular or intracellular amino acids were preferentially used for protein synthesis muscle tissue was preincubated for 2 h at +37 C with labeled ^{14}C leucine (Fig. 4) The tissue was then washed in ice-chilled buffer solution at +4 C for 15 min in order to empty the extracellularly distributed amino acids and those in other compartments without an active transport barrier When the tissue after this wash was

TABLE II Incorporation rate of leucine into human skeletal muscle proteins Mean ± S.E.

Amino acid concentration of the medium	Addition to the incubation medium (mmol l)	Incorporation rate (μ mol leucine h^{-1} g prot^{-1})
Normal plasma level	—	0 055 ± 0 0 5 (5)
10 times plasma level		0 149 ± 0 014 (19)
Normal plasma level	ouabain 0 1	0 053 ± 0 013 (5)
Normal plasma level	mono-iodoacetate 3 0	0 0 5 ± 0 004 (5)
Normal plasma level	puromycin 0 7	0 00 ± 0 000 (3)

P < 0 01 [Wilcoxon's non parametric test (Siegel 1956)]

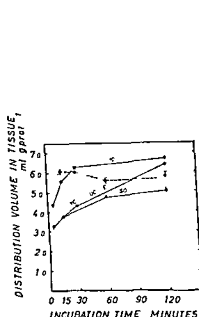


Fig 1

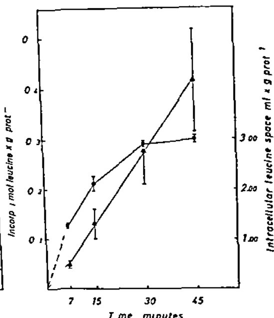


Fig 2

Fig 1 Distribution volume and equilibration time of ^{14}C leucine ^{1} C-cyclo-leucine ^{14}C-sorbitol and $^{3}H_2O$ (O O) Incubat ons and preparations were performed as described under "Material and Methods"

Fig 2 The relationship between the incorporation of leucine into skeletal muscle proteins (▲—▲) and the intracellular d stribution volume of ^{14}C leucine (●—●)

Skeletal muscle fibers ?00 mg were first preincubated for 1 h at 37 C in a medium consisting of a complete amino acid solution at a concentration correspond ng to that of normal human plasma ^{14}C leucine (10^6 DPM) was then added to the incubation medium and the incubations were further run at 37 C in a metabolic shaker (constant agitation 80 cycles/min) as ind cated in the Figure The determination of label in proteins and in the intracellular free amino acid pool were performed as described under "Material and Methods" Mean of 6 determinations is given.

reincubated at +37 C in a new medium containing ^{3}H leucine no further incorporation of ^{1} C leucine was registered On the other hand tritium was immediately and linearly incor porated into proteins before the total pool of intracellular ^{3}H leucine had reached an equi librium The intracellular pool of ^{1} C-leucine was almost replaced by ^{3}H leucine after 30 min incubation However the ^{1} C leucine concentration of the intracellular pool was of a magni tude which if it was preferentially incorporated into proteins should be possible to register in the protein This statement is based on the fact that still after 30 min incubation the specific activity of leucine in the intracellular free amino acid pool was roughly 50 times higher than the corresponding activity in the protein

Discussion

The present studies show that labelled leucine was linearly incorporated into TCA precipit able protein in human skeletal muscle tissue from a point of time before the labelled leucin

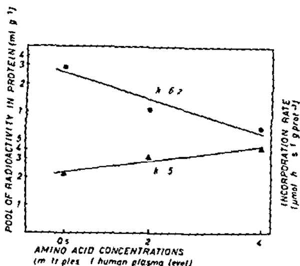


Fig 3 Semilogarithmic plot of the relationship between the labelling of proteins with ¹⁴C leucine and the amino acid concentration in the medium (O—O r=0 88) and the relationship between the incorporation rate of leucine into muscle proteins calculated from the specific activity in the incubation medium and the amino acid concentration in the medium (▲—▲ r=0 93) The calculations were performed from duplicate determinations in the same experiment

had reached an equilibrium with the total intracellular pool of free amino acids If the precursor amino acids were uniformly distributed in the cytoplasm or in the total intracellular pool the appearance of label in protein would be expected to follow a curvilinear function until the total intracellular pool of label had reached equilibrium (Kipnis Reiss and Helmreich 1961) Similar results as ours have been reported by several authors from *in vitro* experiments with different tissue preparations (Kipnis *et al* 1961 Rosenberg *et al* 1963 Roscoe *et al* 1968 Hider *et al* 1971 Adamson Herington and Bornstein 1972, van

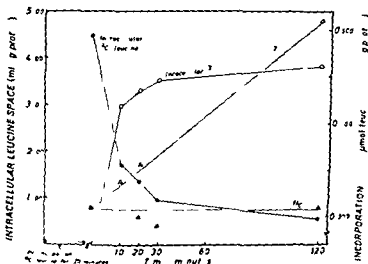


Fig. 4 The incorporation of ³H leucine and ¹⁴C leucine into muscle proteins in comparison with the intracellular space of free ³H leucine and ¹⁴C leucine Muscle fibers 100 mg were preincubated with ¹⁴C leucine and a complete amino acid solution at a concentration corresponding to that of normal human plasma for 2 h After washout for 15 min in a cold KRB buffer solution the fibers were reincubated in a medium containing ³H leucine and amino acids as in the first medium The label in proteins and in the intracellular free amino acid pool was determined as described under "Material and Methods" Each observation represents the mean of 6 determinations

Venrooij *et al* 1972 Stakeberg Gustafson and Scherstén 1974) These experimental findings are incompatible with the assumption that a homogeneous intracellular amino acid pool constitutes the immediate precursor pool for protein synthesis Therefore it has been proposed that extracellular amino acids are preferentially used for protein synthesis *in vitro* (Hider *et al* 1971) However the operationally defined intracellular amino acids in intact *in vitro* preparations may comprise the sum of the amino acids in probably existing different cellular compartments

Sorbitol is not taken up by intact cells to any appreciable extent It is obvious from the results in Fig 1 that ^{14}C-sorbitol attained the intracellular space in our muscle preparation This uptake was probably exclusively via the cut fibre ends (Menozzi *et al* 1959) The ^{14}C sorbitol uptake was as fast as that of ^{14}C-cyclo-leucine but not as fast as that of ^{3}H leucine Since ^{3}H leucine and ^{14}C-cyclo-leucine are actively transported (Lundholm *et al* 1974) this finding indicates the existence of one or several intracellular compartments of ^{3}H leucine and ^{14}C-cyclo-leucine which could be reached only by means of active transport on the intracellular route of the molecules Amino acid transport mechanisms have been shown in isolated mitochondria and nuclei (Allfrey *et al* 1961 Nukada 1965) Cowie (1962) has reported that free amino acids in yeast exist in 2 pools one of which equilibrates readily with amino acids in the medium In the perfused rat liver Mortimore *et al* (1972) have found a compartmentation of free valine consisting of one expandable pool in continuity with protein synthesis sites and one non-expandable pool which failed to equilibrate with the label in the medium From these results it can be concluded that intracellular amino acids are intermediates of protein synthesis which also has been clearly shown by Morgan *et al* (1971)

Mortimore *et al* (1972) calculated the halftime for the equilibration of intracellular valine with the expandable pool in perfused rat liver to 0 96 min In the present study we found that ^{14}C leucine was linearly incorporated into proteins already after 7 min incubation and that about 40 percent of the total intracellular ^{14}C leucine pool had equilibrated at that point of time (Fig 2) This means a maximum value of 0 40 for the ratio between the expandable leucine pool and the total intracellular leucine pool in this muscle preparation This figure is in good conformity with those 0 30–0 40 reported for valine in the perfused rat liver preparation by Mortimore In conclusion all available information seems to be compatible with the hypothesis that there is a rapidly equilibrating intracellular pool of amino acids which is in continuity with the protein synthesis sites

From several *in vitro* (Morgan *et al* 1971 Mortimore Woodside and Henry 1972) and *in vivo* (Gan and Jeffay 1967 Fern and Garlick 1973) studies it is obvious that a varying amount of amino acids from proteolysis is re utilized for protein synthesis Calculated from *in vivo* experiments about 43 percent of the amino acids for muscular protein synthesis was derived from protein catabolism The corresponding value for liver protein synthesis in the rat was 66 percent (Gan and Jeffay 1967 Mortimore *et al* 1972) In preliminary studies we have found that about 15 percent of the amino acid leucine released by proteolysis was reincorporated in human skeletal muscle *in vitro* (to be published) These studies were performed with an incubation medium containing a complete amino acid solution at a concentration corresponding to 10 times that of normal plasma. As illustrated in Fig 3

there was a difference between the regression coefficients for the semilogarithmic plots of the labelling rate of proteins with ^{14}C leucine and the incorporation rate of leucine into proteins calculated from the specific activity in the medium versus the amino acid concentration in the medium in the same experiment This indicates that the specific activity at the immediate precursor site for protein synthesis is different from that of the medium when it was between one and four times that of normal plasma The ratio between the regression coefficients was 1 15 which means that the specific activity was lower in the immediate precursor pool than in the medium (approximately 15%) This phenomenon reflects probably a re utilization of amino acids from proteolysis Hypothetically this underestimation of protein synthesis when calculated from the medium specific activity should be reduced at high medium concentrations of amino acids presumably because of a reduction of the relative importance of the reutilization of label However there was not the expected conformity between labelling of proteins and calculated rate of incorporation at higher substrate levels This discrepancy is difficult to explain but one possibility may be that proteolytically released amino acids are preferentially used for incorporation compared with amino acids in the medium In our experiments the intracellular uptake rate of leucine was almost linear at amino acid concentrations in the medium between 1 and 16 times that of normal plasma Taken together with the rapid equilibration of total tissue space of ^{3}H leucine (Fig. 1) it seems probable that the specific activity of labelled leucine in most tissue pools rapidly approached that of the incubation medium The present findings are compatible with the recently proposed hypothesis of Adamson *et al* (1972) that amino acids proteolytically released intracellularly can return to be once again available for incorporation by reassociation at the transport membrane and that these amino acids have a competitive advantage for incorporation as compared with free intracellular amino acids If this is true *in vitro* determinations of protein synthesis will necessarily give falsely low values inde[illegible]ently of which amino acid pool that is used for the calculations of the synthesis rate However by using a high concentration of amino acids in the medium this error seems to be low An appropriate amino acid concentration for this skeletal muscle preparation may be 10 times that of normal plasma since optimal stimulation for the incorporation of leucine were obtained at that concentration (Lundholm *et al* 1974)

This work was made possible by grants from the Cancer Foundation of Assar Gabrielsson and the Swedish Cancer Society (project No 93)

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Acta physiol scand 1975 93 442-454
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Dynamic Properties of Excitation and Inhibition in the Cochlear Nucleus

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Received 29 July 1974

Abstract

MOLLER A R *Dynamic properties of excitation and inhibition in the cochlear nucleus* Acta physiol scand 1975 *93* 442-454

The dynamic properties of inhibition and excitation of single units in the cochlear nucleus were studied using tones that were amplitude modulat d either sinusoidally or with pseudorandom noise The cross-correlation analysis of the unit discharge rate and the pseudorandom noise modulation of the st muli showed that the dynami properties determined from the responses to a single modulated excitatory tone (at CF) to a modulated excitatory tone together with an unmodulated inhibitory tone (above CF) and to a modulated inhibitory tone together with an unmodulated excitatory tone were almost identical w th regard to latency as well as to delay of the peak of the cross-covariance function On the basis hereof it is inferred that the inhibition is either a cochlear phenomenon or that it is transmitted over pathways with identical temporal properties as those of the excitation When 2 tones were presented sim ltaneously the modulation of the excitatory tone usually gave a higher degree of modulation of the d scharge rate than d d modulation of an inhibitory tone Addition of an unmodulated inhibitory tone to a modulated tone at CF resulted in an extension of the range of intensities over which the maximal gain was relatively constant In many units this range extended from about 10 dB above the unit s threshold to 60 dB or more above

It has been shown in numerous studies that tones excite units in the cochlear nucleus within a limited range of frequency and intensity the threshold having its lowest value at one particular frequency the unit s CF Each unit is thus tuned to a particular frequency That is a typical property of a wide variety of units in the ascending auditory nervous system from primary nerve fibers to single cells in the auditory cortex In many places in the ascending auditory pathway the activity evoked by one tone can furthermore be inhibited by another tone of suitable frequency and intensity In the cochlear nucleus such inhibition occurs regularly (See *e g* Galambos 1944 Rose *et al* 1959 Greenwood and Maruyama 1965 Gerstein *et al* 1968 Starr and Britt 1970) A tone with a frequency slightly higher than the upper limit of the response area is usually the most efficient inhibitory stimulus but inhibition also occurs in many units in the frequency range below the excitatory response area Each such unit thus has an excitatory area in response to pure tones which is "sand

wiched between two inhibitory areas The arrangement of inhibitory and excitatory areas in the cochlear nucleus units is such that most natural sounds which usually have their energy distributed over a wide range of frequencies are likely to activate both inhibitory and excitatory areas in many units simultaneously Since natural sounds furthermore usually vary more or less rapidly both in amplitude and in spectral composition it is of great interest to study the dynamic properties of both inhibitory and excitatory sounds

It has been shown earlier (Møller 1972, 1973) that sinusoidal amplitude modulation of a tone in a certain range of modulation frequencies gives rise to a comparatively large modulation of the discharge rate of single units in the cochlear nucleus The ratio between the relative frequency modulation of the discharge rate and the relative modulation of the amplitude of the stimulus tones varies as a function of the modulation frequency by definition, the *transfer function* In many units this transfer function has a more or less pronounced peak at a certain modulation frequency The frequency of this peak varies from unit to unit between 50 and 300 Hz. In many units this peak becomes more pronounced as the stimulus sound intensity is increased while in other units the shapes of the transfer functions are nearly unchanged in a large sound intensity range (Møller 1972) In nearly all the units studied modulation in frequencies below 10 or 20 Hz was shown to be attenuated when the stimulus intensity was increased while the modulation of the discharge rate in the range from 50–300 Hz was preserved in most units over a large range of intensities (Møller 1974 a)

In previous studies it has been shown that linear systems analysis can be applied in the estimation of the frequency response function of cochlear nucleus units in response to amplitude modulated tones and noise (Møller 1973) In these studies the tones or noise were modulated either sinusoidally (Møller 1972) or with pseudorandom noise (Møller 1973) The dynamic properties were described by means of transfer functions Such transfer functions express the ratio between relative modulation of the discharge density and relative modulation of the tone stimuli as a function of modulation frequency with regard to both amplitude and phase angles When sinusoidal modulation was used the transfer functions were evaluated from cycle histograms of the responses locked to the modulation signals The use of noise modulated sound stimuli made it possible to determine the transfer function in a large range of modulation frequencies on the basis of one single recording With this method the transfer function is computed from the cross-covariance between the modulation of the sound and the modulation of the discharge rate Furthermore noise modulated sounds resemble natural sounds to a larger extent than do sinusoidally amplitude modulated sounds

That there is a high degree of linear relationship between the envelope of a sound and the discharge rate is furthermore shown by the fact that the transfer functions determined on the basis of the cross-covariance between the noise used as modulation and the neural discharge frequency are very similar to those obtained using sinusoidal modulation at discrete frequencies (Møller 1973)

Natural sounds with their usually more or less wide spectral distribution are likely to activate both inhibitory and excitatory areas of many units simultaneously Such sounds usually vary in amplitude and spectral composition more or less rapidly In order

knowledge about the coding of natural sounds it is therefore important to study the mutual interaction between excitatory and inhibitory sounds and the way in which changes in the amplitude of such sounds are coded.

In the previous studies one single amplitude modulated tone the frequency of which was equal to the CF of the unit was used. It is therefore not known whether the dynamic properties of the inhibition differ from those of excitation.

In the present study tones, amplitude modulated with sinusoids or pseudorandom noise were used to estimate the transfer function from modulation of a tone to unit discharge rate (spike density) for a single tone at the unit's characteristic frequency (CF) as well as for 2 tones presented simultaneously, one excitatory tone at CF and one inhibitory tone at the best inhibitory frequency (BIF). One of the 2 tones was modulated at a time. In the case of noise modulated tones transfer functions were computed from the cross-covariance between the variation in spike density and the modulation of the sounds.

Methods

Rats weighing 200–300 g were anesthetized with urethane (initial dose of 1.5 g/kg b.wt.). The surgical procedure and recording technique have been described previously (Møller 1969, 1971, 1973).

Stimuli consisted of tones, the amplitude of which was modulated either sinusoidally or by pseudorandom noise generated by a Hewlett Packard noise generator type 3722A. A noise sequence length of 136 ms (4095 points with a clock period of 33.3 µs) was used. The bandwidth of the noise was 1500 Hz and its peak factor was 3.75. Pure tones were generated by Wavetek (type 11[illegible]) generators and the amplitude modulation was performed by means of a Motorola (MC 1595 L) 4 quadrant multiplier.

Sinusoidally modulated tones were usually presented during 15 s followed by 15 s of silence. Noise modulated tones were presented according to the same scheme with a one-to-one ratio of pause to tone and the responses were averaged over 5 or 10 min. The frequencies of the stimulus tones were continuously monitored by digital frequency meters.

Cycle histograms of the responses to sinusoidally modulated tones were made by an Intertechnique Physioscope DIDAC 800. The output of the analyzer was punched on paper tape and read into a general purpose computer (IBM 360/75). A sine wave was matched to the histograms and its amplitude and phase angle were used to determine the gain functions (see Møller 197[illegible]).

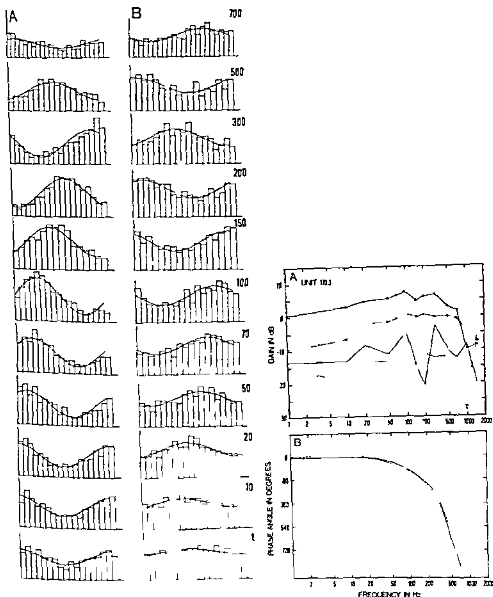
The responses to pseudorandom noise were processed in a similar way and cycle histograms locked to the periodicity of the noise were made on line using the DIDAC 800. Its output was punched on paper tape for further processing on the general purpose computer. The data processing used comprised computation of cross-covariance between the histograms and the modulation. On the basis hereof the transfer functions (gain functions) were computed. The details of the data processing have been given earlier (Møller 1973 and 1974 b).

Results

The results of the present study were based on recordings from 32 units in 18 rats. Several of the units included were kept in good condition during 4 h or more. Only units which could be so maintained for 1 h or more are included.

Responses to sinusoidally amplitude modulated tones

Fig. 1 shows cycle histograms of the response to stimulation with 2 tones presented simultaneously, one of which was sinusoidally amplitude modulated. The frequency of one of the tones was equal to the unit's characteristic frequency (CF) and that of the other was the unit's best inhibitory frequency (BIF). In A the excitatory tone was modulated while



F g 1 Cycle h stograms of the responses to stimulat on with tones one of wh ch was amplitude modul ated A Modulation of the e citatory tone B Modulatio of the inhibitory tone The frequency of the excitatory tone was equal to the unit s CF (4 500 Hz) and its intensity was 55 dB SPL The frequency of the inh b tory tone was qu l to the un t s best inhibitory frequency (5 500 Hz) and its intensity was 65 dB SPL.

Fig Relative ga n (A) and phase angle (B) of a sine wave matched to fit the cycle h tograms of the responses to 2 tones one of wh h was sinuso dally amplitude mod lated Lines w th symbols nd cate the fundamental wa e a d lines w tho t symbols show the relati e gain of the second harmon wave Sol d l nes represent modulation of the e citatory tone and dashed l nes mod lat on of the inhib tory tone Phase a gles are shown only for the fundamental wave The curve for modulation of the inhib tory to e was shifted 180 to f c l t t comparison

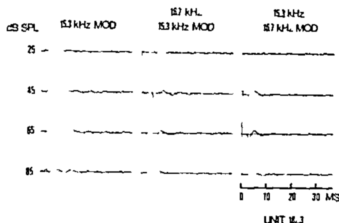


Fig 3 Cross-covariance functions computed from responses to pseudorandom noise modulated tones Left column One single tone at CF middle and right columns 2 tones one excitatory at CF and one inhibitory at BIF Middle column Modulation of the excitatory tone Right column Modulation of the inhibitory tone The intensities of the excitatory tone are indicated by legend numbers The intensity of the inhibitory tone was equal to that of the excitatory

the inhibitory tone was unmodulated and in B vice versa The intensity of the excitatory tone was 55 dB SPL and that of the inhibitory tone was 65 dB SPL The unit s threshold at CF was 30 dB SPL

It is seen that the modulation of the histograms in all cases resembles a sine wave When the inhibitory tone is modulated the phase of the sine wave modulation of the histograms is reversed compared with modulation of the excitatory tones When the inhibitory tone is modulated the degree of modulation of the histograms is smaller than when the excitatory tone is modulated

The transfer functions computed from the histograms in Fig 1 are seen in Fig 2 A It is seen that the relative gain (ratio in dB between the relative modulation of the histogram and that of the stimulus tone) varies as a function of modulation frequency in a similar way in the two different situations i e when the excitatory tone is modulated (solid line) and when the inhibitory tone is modulated (dashed line)

Fig 2 A also shows the content of the second harmonic in the cycle histograms (dashed and solid lines without symbols) It is seen that the distortion has about the same value for inhibition and excitation Fig 2 B shows the phase angle between the modulation of the tones and that of the histogram when the excitatory tone was modulated (solid lines) and when the inhibitory tone was modulated (dashed lines) The latter curve was shifted 180 to facilitate comparison

The results thus indicate that the dynamic properties of excitation and inhibition are similar in these units That was a typical finding and it was further studied in a number of units by applying tones modulated with pseudorandom noise

Response to pseudorandom noise modulated tones

When the input to a linear system is random noise with uniform spectrum in the frequency range within which the system has a significant transmission the cross-covariance function between the input and output is a valid approximation to the impulse response of the system i e the output of the system in response to an infinitely short excitation The cross-covariance functions are functions of delay between input and output and the impulse responses are



Fig. 4 Same as Fig 3 but for another unit The intensity of the inhibitory tone was 4 dB higher than that of the excitatory tone

functions of time Consequently the transfer function of a linear system showing the system s gain (i e ratio between output and input) as a function of frequency can be computed on the basis of the computed cross-covariance functions In linear systems direct determination of the system s impulse response has its own value since it may add to the understanding of the function of the system In nonlinear systems the response to one type of stimulation cannot necessarily be deduced on the basis of the results obtained using other types of stimulation

As input noise has many advantages over both sinusoidal input and short impulses particularly in systems which show weak or moderate nonlinearity like most biological systems The properties of such systems can in many cases be approximated to those of a linear system with regard to small variations around a certain operating point

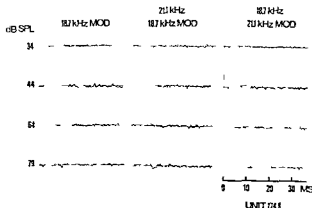


Fig 5 Same as F g 3 but for another un t The intensity of th inhib tory tone was equal to that of the excitatory to e

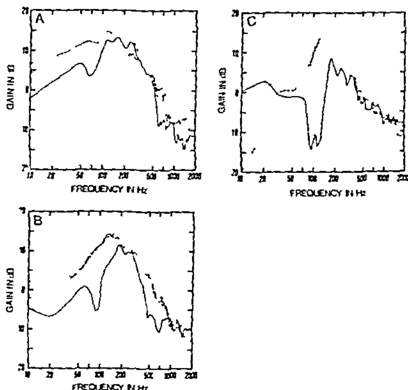


Fig. 6 Transfer functions computed from the cross-covariance functions of Fig 4 at 3 different sound intensities. In A the intensity of the excitatory tone was 30 dB SPL, in B it was 50 dB and in C 70 dB SPL. The intensity of the inhibitory tone was 4 dB higher than that of the e citatory tone Sol d lines represent modulat on of one single excitatory tone (left column in Fig. 4) dashes ind cate stimulation with tones and modulation of the inhibitory tone (right column in Fig. 4) dashes and dots denote modulation of the excitatory tone in the presence of an unmodulated excitatory tone (middle column in Fig. 4)

In the present study pseudorandom noise was employed instead of ordinary noise for practical reasons (Moller 1973 1974 b) The amplitude modulation of a tone is regarded as the input of the system to be studied and the output is modulation of the neural discharge rate (spike density)

Fig. 3 shows typical cross-covariance functions obtained from the responses to tones modulated with pseudorandom noise at various stimulus intensities The left column shows results obtained when a single amplitude modulated tone at CF was presented the middle and right columns show results based on the responses to two tones one at the unit s CF (15 3 kHz) and one at its best inhibitory frequency (BIF) (16 7 kHz) One of the two tones was amplitude modulated with pseudorandom noise and one was unmodulated The intensity of the 2 tones indicated by legend numbers was the same The amplitude scales of the cross-covariance functions are normalized to represent the absolute modulation of the neural discharge frequency The degree of modulation was 13 dB re 100 RMS modulation It is seen that the cross-covariance functions are reversed but similar in shape when the inhibitory tone is modulated compared with the situation when the excitatory tone is modulated The peak amplitude of the cross-covariance function is greater when two

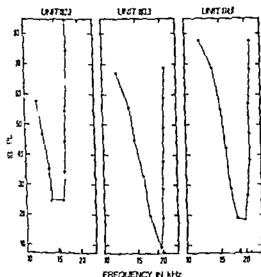


Fig 7 Tuning curves of the units depicted in Fig 3–5 The threshold was determined in responses to 50 ms long tone bursts

tones are presented simultaneously and the excitatory tone is modulated compared with the situation when a single excitatory tone is modulated (except for the sound intensities near threshold) The peak value of the cross-covariance has its lowest value when the inhibitory tone is modulated in the presence of an unmodulated excitatory tone (C) There is also a change in shape of the cross-covariance as the stimulus intensity is increased Comparison between the responses when the excitatory and inhibitory tones are modulated shows that aside from being reversed, the cross-covariance functions are remarkably similar in shape It should particularly be noted that there is no discernible difference in the latency of the cross-covariance functions for modulation of the excitatory or inhibitory tone nor is there any detectable difference in delay of the occurrence of the peak amplitude of the cross-covariance Fig 4 and 5 show similar recordings from 2 other units revealing results which are essentially identical to those shown in Fig 3 Fig 6 shows the corresponding transfer functions computed from the cross-covariance functions of the responses to tones amplitude modulated with pseudorandom noise shown in Fig 4 for various sound intensities The relative gain (ratio between the relative modulation of the discharge rate and the relative amplitude modulation of the stimulus tones) is shown as a function of modulation frequency The transfer functions shown in Fig 6 represent modulation of a single excitatory tone (solid lines) modulation of an excitatory tone when an inhibitory tone of the same intensity was added (dashes and dots) and modulation of an inhibitory tone while the excitatory remained unmodulated (dashed lines) It is seen that the transfer functions are similar in shape in all the three different experimental situations but that the gain has a lower value when modulation of the inhibitory tone is involved than when modulation of the excitatory tone is performed

The tuning curves of the 3 units depicted in Fig 3–5 are seen in Fig 7 with t of the excitatory tone indicated by filled arrows and that of the inhibitory tone in

Fig. 8. Post stimulus time histograms of the responses of the 3 units depicted in Fig. 3-5. Stimulation was 50 ms long tone bursts, the intensity of which is indicated by legend numbers. Each histogram represents [illegible] stimulus presentations. Heavy lines indicate stimulus duration.

by open arrows. Fig. 8 shows PST histograms of the responses to 50 ms tone bursts at various intensities (indicated by legend numbers) for the 3 units depicted in Fig. 3-5. The corresponding stimulus response curves showing discharges per second as a function of sound intensity are seen in Fig. 9.

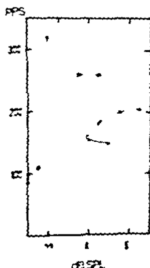


Fig. 9. Stimulus response curves of the 3 units depicted in Fig. 3-5 in response to 50 ms tone bursts showing discharges per second during the sound presentation. Solid lines and circles: Unit 183.3; dashes and dots and squares: Unit 182.3; dashes and triangles: Unit 174.8. The curves are based on the data in Fig. 8.

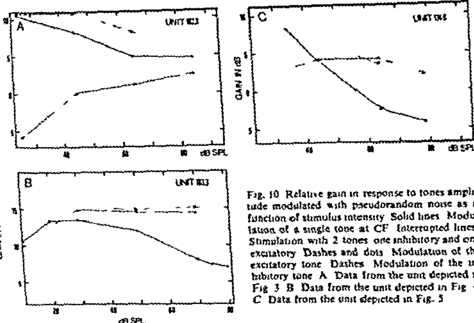


Fig. 10 Relative gain in response to tones amplitude modulated with pseudorandom noise as a function of stimulus intensity Solid lines Modulation of a single tone at CF Interrupted lines Stimulation with 2 tones one inhibitory and one excitatory Dashes and dots Modulation of the excitatory tone Dashes Modulation of the inhibitory tone A Data from the unit depicted in Fig 3 B Data from the unit depicted in Fig 4 C Data from the unit depicted in Fig. 5

The results shown in Fig 1–6 are typical for the units from which recordings were made originating from both the dorsal and the ventral cochlear nucleus Just as has been shown earlier (Møller 1972, 1973) the value of the gain and the shape of the gain function may differ from unit to unit In none of the units with CF ranging from 1 kHz to 40 kHz were there any detectable differences in the latency of the cross-covariance when the excitatory tone was modulated compared with when the inhibitory tone was modulated Nor was there any discernible difference in the delay at which the peak value of the cross-covariance occurred In some units there were slight differences in the shape of the cross-covariance when the inhibitory tone was modulated compared with when the excitatory tone was modulated Corresponding differences in the computed transfer function were seen The peak in the transfer function however occurred at nearly the same frequency in all three stimulus situations,

Relation between maximal gain and stimulus intensity

The relationship between the maximal gain (the relative gain at the modulation frequency where it has its highest value) and the sound stimulus intensity is graphed in Fig 10 for the 3 units illustrated in Fig. 3–5 Values are entered for all 3 experimental situations a single modulated tone of CF solid lines a modulated excitatory tone in the presence of an inhibitory tone dashes and dots and modulation of the inhibitory tone in the presence of an unmodulated excitatory tone dashed lines

It is seen that the modulation of an excitatory tone in the presence of an unmodulated inhibitory tone results in a higher gain than modulation of a single excitatory tone over

Fig. 8 Post stimulus time histograms of the responses of the 3 units depicted in Fig. 3–5 Stimulation was 50 ms long tone bursts the intensity of which is indicated by legend numbers Each histogram represents 250 stimulus presentations Heavy lines indicate stimulus duration

by open arrows Fig 8 shows PST histograms of the responses to 50 ms tone bursts at various intensities (indicated by legend numbers) for the 3 units depicted in Fig. 3–5 The corresponding stimulus response curves showing discharges per second as a function of sound intensity are seen in Fig 9

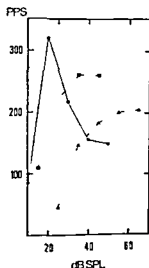


Fig. 9 Stimulus response curves of the 3 units depicted in Fig 3–5 in response to 50 ms tone bursts showing d scharges per second during the sound presentation Solid lines and circles Unit 183 3 dashes and dots and squares Unit 18 3 dashes and triangles Unit 174 8 The curves are based on the data in Fig 8

range of intensities where changes in amplitude of an excitatory tone are converted into corresponding modulation of the discharge frequency in single neurons in the cochlear nucleus

The fact that the temporal characteristics of inhibition and excitation are nearly identical shows that the inhibition is accomplished in very much the same way as the excitation and not through any additional interneurons One can hence assume that the inhibition of the cochlear nucleus neurons from which recording has been made is accomplished as a result of the suppression seen in primary neurons (Nomoto *et al* 1964 Sachs and Kiang 1968) or that these cochlear nucleus neurons have 2 different types of synapses one inhibitory and one excitatory the input to which consists of primary fibers from haircells along the basilar membrane of the cochlea

The fact that an inhibitory sound can reduce the firing rate evoked by an excitatory sound may be responsible for the extended range of intensities over which the modulations of amplitude modulated sounds are coded in the discharge frequency

Recent studies by Evans and Nelson (1973 a b) have shown that anesthesia causes marked changes in responses from many cells in the dorsal cochlear nucleus among which is a reduction in inhibition Preliminary studies in the unanesthetized rat at our laboratories using the above described technique have confirmed that neurons with more complex inhibitory patterns exist in the dorsal cochlear nucleus These preliminary studies also showed that units with a primary like response had a similar response pattern in the unanesthetized rat as in the anesthetized rat thus in agreement with the findings by Evans and Nelson It is concluded that the response patterns obtained in the anesthetized preparations are representative for neurons in the ventral cochlear nucleus and hence perhaps in the dorsal cochlear nucleus

The results of the present study indicate that small and rapid changes in amplitude of a spectral component of a complex sound are likely to be coded as a much larger modulation of the discharge rate than can be inferred from the steady state responses to pure tones Furthermore such modulation is preserved at a relatively constant level over a stimulus intensity range that is far larger than the range where steady state or slow changes in intensity are coded

The conclusion of the present results is that the dynamic range of the neurons in the cochlear nucleus is extended when 2 tones are presented simultaneously compared with the range appearing under stimulation with a single tone Moreover the sensitivity for rapid changes for 2 tones is maintained at nearly the same level over a large range of intensities as it is for weak single pure tones It is on the basis hereof hypothesized that one important role of inhibition is to make it possible to keep a high sensitivity to small rapid changes in amplitude over a large range of sound intensity hence avoiding the need of a large range of static discharge rates

This work was supported by the Swedish Medical Research Council (Projects Nos 04-X 90 and P 3 51) and by Johan and Therese Anderson s St fielsen Computer t me was made available by the Karolinska Institute Th author is ndebt d to Pamela Boston for read ng the manuscript Carina Erickson ass sted n the experiments and prepared the figures togethe with Ingrid Moller The v luable help is greatly acknowledged

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Acta physiol scand 1975 93 455–465

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Consequence of Social Isolation on Blood Pressure, Cardiovascular Reactivity and Design in Spontaneously Hypertensive Rats

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Received 17 September 1974

Abstract

HALLBÄCK M *Consequence of social isolation on blood pressure cardiovascular reactivity and design in spontaneously hypertensive rats* Acta physiol scand 1975 *93* 455–465

Spontaneously hypertensive rats (SHR) which inherently display exaggerated cardiovascular defence reactions to environmental stimuli (Hallbäck and Folkow 1974) and normotensive control rats (NCR) were kept isolated after they were weaned to reduce such environmental influences which normally induce psychological activation Mean arterial pressure was followed until 7 months of age when the cardiovascular defence reactions to acute mental stress were compared and an analysis of cardiovascular design was made The isolated SHR but not the isolated NCR had significantly lower pressures than their unisolated controls Likewise judged by the relative weight of the left ventricle and the hemodynamically evaluated design of the hindquarter resistance vessels the structural cardiovascular adaptation was about proportionally less pronounced in isolated than in control SHR However their cardiovascular responses to acute "psychological stress" were equally intense and clearly exaggerated when compared with NCR Thus a prolonged reduction of excitatory environmental influences implies a relatively less pronounced development of hypertension in SHR even though an inherent hyperreactivity concerning neurohormonal pressor responses to alerting stimuli is present. These findings stress the importance of interacting intrinsic hereditary and extrinsic neurogenic influences for the initiation of primary hypertension

Spontaneously hypertensive rats (SHR Okamoto 1969) exhibit exaggerated cardiovascular responses *i e* more pronounced defence reactions than normotensive control rats (NCR) when both are exposed to different levels of "psychological stress" (Hallbäck and Folkow 1974) This centrally integrated response pattern also normally occurring in man when alerted (Brod *et al* 1959) includes an increased sympathetic discharge to heart and most vascular beds but is often associated with a neurogenic vasodilatation in the skeletal muscles Also young pre hypertensive SHR show exaggerated cardiovascular responses when stress-stimulated and, in general, such responses seem to be more easily elicited than in NCR. However renal hypertensive rats representing a non hereditary type of hypertension do not react differently from ordinary NCR in this respect Such findings indicate that the genetic background, and an inherent hyperreactivity rather than the high blood pressure *per se* forms

background of the enhanced defence reactions in SHR It also follows that frequent and exaggerated defence reactions in response to daily alerting stimuli tend to raise the *average* blood pressure level over days and weeks as a result of their intermittent pressor influences Such an increased average load on heart and resistance vessels will gradually initiate adaptive structural changes where particularly the changes of the precapillary resistance vessels seem to be of key importance for the establishment of hypertension (*cf* Folkow *et al* 1973 1974) Furthermore a persistent increase in blood pressure has been found to occur also in genetically normotensive animals following chronic exposure to stress stimulation (Hudak and Buckley 1961 Herd *et al* 1969 Smookler *et al* 1973) Further, when the hypothalamic defence area which induces and integrates the cardiovascular responses during stress is intermittently stimulated *via* implanted electrodes for some months in rats their resting blood pressure levels slowly rise when compared with similarly implanted but unstimulated control rats (Folkow and Rubinstein 1966)

As mentioned above SHR seems for genetical reasons to display defence reactions more easily than genetically normotensive rats when exposed to alerting stimuli As a consequence more frequent and more pronounced psychogenic cardiovascular excitations are likely to occur in SHR compared with NCR also during everyday life since the habitual cage environment quite likely includes frequent episodes of arousal for the rats during various types of social confrontations In order to investigate the importance of such *per se* normal social interactions upon the development of primary hypertension in SHR male SHR and NCR were parted from social confrontations from the time of weaning Thus the rats were isolated one by one in separate boxes but otherwise without any interference of daily routine Mean arterial pressure of the isolated SHR and NCR and also that of 2 control groups of unisolated brothers was intermittently recorded up to the age of 7 months At s age the cardiovascular responses to acute psychological stress were investigated in paired experiments as were the changes in heart weight and in structural design of the resistance vessels in the two SHR groups in comparison with normotensive controls

Methods

At weaning *i* at the age of 28 30 days 9 male SHR and 10 male NCR were placed one by one in isolation cages while brothers serving as controls were housed 6 by 6 in a usual manner The isolation cages ordinary plexiglass cages sized 35 0 15 cm and the cages containing the controls sized 40 30 0 cm were placed in the same animal room and all animals were submitted to the same daily care On four occasions at the age of 1½ 4 5½ and 7 months blood pressure was measured intraarterially in a randomized order by the same technician in these 4 groups of rats when awake (see Weiss 1974) At the age of 7 months the isolated SHR (SHR_I) and the control SHR groups *i e* those housed 6 by 6 (SHR_C) were exposed to acute psychological stress in paired experiments with control NCR The tail arteries of one SHR (SHR_I or SHR_C) and one matched NCR were cannulated during brief ether anesthesia The incision was sutured and local anesthesia was administered The rats were then placed in a special two-compartment stimulation box with one rat in each compartment The tail cannulas were connected to Statham pressure transducers writing on a Grass polygraph no 7 for continuous blood pressure recordings Heart rate was also continuously followed by means of a tachygraph triggered by the systolic pressure rise

After 1 h of recovery from anesthesia and acclimatization to the new environment the 2 rats were exposed to sudden stimulation with loud noise for 30 s and after a 15 min rest period to sudden vibration of the box for 30 s The responses to these stimulations in terms of percentage change in heart rate from resting level and absolute change in blood pressure were analyzed with a Hewlett Packard Calculator

98 0A combined with a digitizer unit 9864 The individual response of each animal and the difference in response between each pair of SHR NCR were calculated for each 3 s interval during the 30 s of stimulation and the following 30 s The means ±S E for each group as well as the mean differences ±S E between SHR_I and their NCR or between SHR_C and their NCR were then calculated for each third second interval The level of significant difference was calculated according to the pairing design t test A significant difference in response to stress stimulations between groups was considered to be present when 1. of the 20 mean values during the 60 s period had a probability of equality of less than 0 05 The 2 SHR groups could then be statistically compared for each 3 s interval value *via* their difference to their matched NCR by means of group comparison t test For further details see Hallback and Folkow 1974

For hemodynamic evaluation of the design of the resistance vessels pairs of isolated hindquarters from SHR_I-NCR or from SHR_C-NCR were simultaneously perfused with an artificial plasma substitute as pre iously described in detail (Folkow *et al* 1970 Lundgren 1974 Weiss 1974) When the perfusion had started the hearts were ext rpated and the left ventricles carefully dissected and weighed The vascular beds of the hindquarters were initially perfused during maximal vasodilatation (ensured by slug injections of papaverine 0 5 1 5 mg) at different flow rates while the perfusion pressure was recorded *r a* the tail artery Thereafter flow was kept constant at a level of approximately 8 ml/min × 100 g tissue during which increasing doses of noradrenaline (NA) were infused To ensure definitely max mal pressor re sponses vasopressin 10 IU and $BaCl_2$ 150 mg were given after maximal NA responses had been achie ed Dose response curves to NA *i e* resistance curves were then constructed for each pair of rats (for details concerning the res stance curves see Folkow *et al* 1970 Lundgren 1974 Weiss 1974) Statistical analysis of the mean values of the different key points of these curves were performed according to the pairing design t test when each group of SHR was to be compared with their pa red NCR For compari sons between SHR_C and SHR_I their respective differences to NCR were compared according to the group comparison t test Further d fferences in blood pressure heart rate left ventricular weight and body weight were analysed statistically by means of group comparison t test

Results

I *Blood pressure changes* After 2 weeks of isolation at the age of 1 5 months mean arterial pressure of the two SHR groups was equal *i e* 111 ±2 mm Hg At this age the NCR groups had a blood pressure of 98 ±3 which was significantly ($p < 0\ 001$) lower than that of SHR remaining so throughout the 7 months of investigation After 3 months of isolation at the age of 4 months and throughout the remaining time of investigation SHR_I had significantly ($p < 0\ 01$) lower blood pressures than their unisolated brothers *i e* SHR_C (see Fig 1 and Table I) However no difference in blood pressure level between NCR_I and NCR_C could be demonstrated at any time during the investigation period

II *Responses to alerting stimuli* When exposed to acute stress stimulation (loud noise or vibrations) SHR_I and SHR_C responded with significantly more pronounced heart rate responses than their NCR. The heart rate increase during the defence reaction depends both on an increased sympathetic and a decreased vagal tone to the heart (*cf* Hallback and Folkow 1974) Due to the close relationship between change in autonomic fibre discharge and change in heart rate (Folkow Lofving and Mellander 1956 Carlsten Folkow and Hamberger 1957) the per cent increase in heart rate was utilized as an indicator of shifts in neurogenic drive The mean peak increase in heart rate in the 2 SHR groups did not differ significantly *i e* 16 ±3 per cent and 20 ±3 per cent increase from resting heart rate in SHR_I and SHR_C respectively while NCR showed a mean peak response of only 9 ±3 per cent The resting heart rate of SHR_I (343 ±10 beats/min) and that of SHR_C (337 ±10 beats/min) did not differ but both groups showed significantly ($p < 0\ 01$) lower rates than NCR (374 ±8 beats/min) These results are in agreement with pre

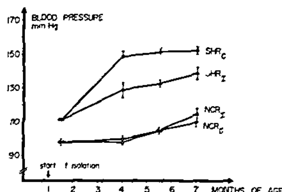


Fig 1 Levels of mean arterial pressure ($\pm$S E) for SHR_C (n = 10) SHR_I (n = 9) NCR_I (n = 10) and NCR_C (n = 10) from 1.5 up to 7 months of age as measured on 4 different occasions while awake and at rest.

of a reduced resting heart rate in SHR due to a reduced intrinsic heart rate but with an increased sympathetic to the heart and decreased vagal influence in SHR as compared to NCR (Hallback and Folkow 1974)

Fig. 2 right side shows the mean difference in heart rate response for each third second interval between SHR_I–NCR (upper panel) and between SHR_C–NCR (lower panel) The responses of SHR_I and SHR_C were then statistically compared via their differences to the respective NCR i e (SHR_I–NCR) minus (SHR_C–NCR) According to the group comparison t test only one observation differed significantly between SHR_I–NCR and SHR_C–NCR and thus the two SHR groups were considered to have responded with equally pronounced tachycardias during the periods of graded stress stimulations (see Methods)

Concerning blood pressure changes during stimulation the mean *peak* responses of SHR_C (24 $\pm$ 3 mm Hg) and that of SHR_I (18 $\pm$ 2 mm Hg) were of the same order of magnitude but both were considerably higher than that of NCR (8 $\pm$ 2 mm Hg) Concerning the average pressure rise during the period of stimulation and the 30 s poststimulatory period Fig 2 left side shows that SHR_I but not SHR_C responded with more pronounced average increases in pressure than their paired NCR However when comparing SHR_I with SHR_C via their respective differences to NCR concerning blood pressure responses (left side of Fig. 2) only 7 of the 20 observations differed significantly ($p < 0.05$) Therefore SHR_I and SHR_C were not considered to show any real difference concerning average pressure responses during the periods of stress stimulation if anything the responses tended to be less pronounced in SHR_C

Hence the cardiovascular responses to acute psychological stress did not differ between SHR_I and SHR_C It is therefore suggested that the intensity of the defence reaction was about equal in the two SHR groups while both showed significantly more pronounced reactions than NCR

III *Hemodynamic evaluation of structural design of the resistance vessels* The pressure flow relationships and the resistance curves obtained from the perfusion analysis of the hindquarter preparations exhibit a number of specific key points" from which differences in resistance vessel design can be evaluated In previous studies the relevance of using these key points has been thoroughly discussed (Folkow *et al* 1970 Lundgren 1974 Weiss 1974)

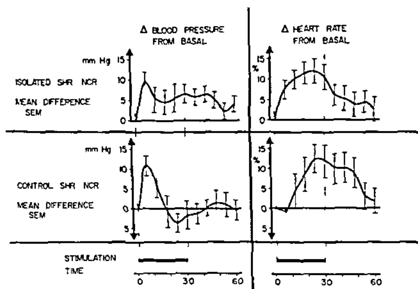


Fig Mean differences (±S E) in blood pressure (left) and heart rate (right) responses to stress stimulation (exposure to sudden noise and vibration) between 9 pairs of SHR_I-NCR_C (upper panel) and 10 pairs of SHR_C-NCR_C (lower panel)

Thus resistance at maximal dilatation can be considered to be inversely related to the *structurally determined bore of the resistance vessels and when measured at different* pressure levels differences with respect to their wall distensibility can also be evaluated The tangent of the angle of the resistance curve *i e* the curve steepness is particularly dependent on the wall/lumen ratio of the resistance vessels while the maximal pressor response is a reflection of their maximal contractile strength The maximal pressor response under the circumstances it is induced in this analysis is therefore dependent on the bulk of vascular smooth muscle in relation to the lumen

Table I presents the mean values ±S E for the arterial blood pressure at the time of acute experiments for the 3 groups as well as the values for the above mentioned key points of the resistance curves together with the levels of significant difference between groups Based on the values in Table I compiled resistance curves were constructed for the 3 groups of rats and are presented in Fig 3 The resistance curve for SHR_C shows an increased resistance at maximal dilatation an increased steepness of the resistance curve and an increased maximal pressor response as compared to NCR. This is also the case for the resistance curve of SHR_I although to a less pronounced degree than in SHR_C This strongly suggests that the resistance vessels of SHR as compared to NCR have an increased wall/lumen ratio with a relative increase in media thickness encroaching upon the lumen also during maximal dilatation This adaptive change in resistance vessel design is however less pronounced in the SHR_I than in the SHR_C as judged by the significantly lower maximal pressor response and the less steep slope of the resistance curve in SHR_I

IV *Structural adaptation of the heart* The percentage of left ventricular weight p

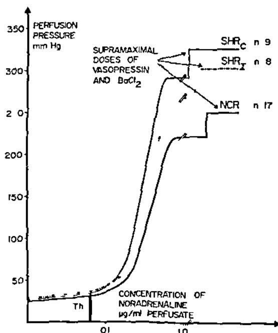


Fig 3 Average pressure (resistance) changes in constantly perfused hindquarter vascular beds of SHR_C SHR_I and NCR_C from the level of maximal vasodilatation up to maximal constriction as induced by increasing nor adrenaline (NA) concentrations with final addition of $BaCl_2$ and vasopressin These compiled resistance curves are based on the values in Table I where the key points of the curves are presented together with S E Th which indicates the threshold NA dose *i e* that concentration which increases resistance by 25 per cent beyond that at maximal dilatation did not differ between groups.

weight was taken as a parameter of cardiac adaptation to the increased pressure load Since the body weights of both SHR_I (421 ±8 g) and NCR_I (437±11 g) were significantly higher than that of the SHR_C (380±7 g) and NCR_C (378±6 g) and since the per cent heart weight per body weight decreases with increasing body weight 5 rats from each group with similar body weights were selected for comparison (Table II) As seen from this table the degree of

TABLE I Mean values (±S E) and mean differences (±S E) within the groups in 8 pairs of SHR_I-NCR_C and 9 pairs of SHR_C-NCR_C for 1) mean arterial pressure 2) hindquarter flow resistance at maximal vasodilatation (PRU_{100} at 30 ml of flow per min and 100 g) 3) slope of the steep part of the NA dose response curves (tangent of the angle) and 4) maximal pressor response The two SHR groups are also compared *via* their mean differences to the respective NCR and the levels of significant differences are gi en

	Arterial blood pressure mm Hg	PRU 00 at max dil	Tangent of the angle	Maximal pressor response mm Hg
SHR_I	139±4	1 99±0 09	4 9±0 5	30 ±10
NCR	108±2	1 75±0 09	3 8±0 4	246± 8
Mean difference	32±4	0 25±0 16	1 0±0 6	56± 7
Significance	p<0 001	n s	n s	p<0 001
SHR_C	15? ±2	1 84±0 06	5 9±0 5	3 6±7
NCR	108±2	1 63±0 05	3 7±0 4	-50±8
Mean difference	45±3	0 ±0 05	?.2±0 5	77±6
Significance	p<0 001	p<0 001	p<0 001	p<0 001
Mean difference SHR_I-NCR versus Mean difference SHR_C-NCR Significance	p<0 01	n s	p<0 05	p<0 05

TABLE II Mean values (±S E.) for body weight mean arterial pressure left ventricular weight, and percentage left ventricular weight/body weight, in 5 SHR_I 5 SHR_C and 5 NCR_C which were closely similar in body weights

	Body weight, g	Arterial blood pressure mm Hg	Left ventricular weight mg	Percentage left ventricular weight/body weight
SHR_I	404±9	139±4	1 025±34	0 255±0 008
SHR_C	40[illegible]±9	152±2	1 103±33	0 [illegible]75±0 006
NCR	*403±6*	*108±*[illegible]	*699±13*	*0 171±0 004*
Level of significance between				
SHR_I/SHR_C	$p<0 4$	$p<0 01$	$p<0 05$	$p<0 05$
SHR_I/NCR	$p<0.5$	$p<0 001$	$p<0 001$	$p<0 001$
SHR_C/NCR	$p<0 5$	$p<0 001$	$p<0 001$	$p<0 001$

left ventricular hypertrophy is closely balanced to the respective pressure levels in SHR_I and SHR_C

Summarizing the present results prolonged social deprivation which had no effect on the blood pressure level in NCR, reduced the progression of hypertension in SHR despite the fact that it did not affect their exaggerated cardiovascular responses to acute psychological stress Furthermore it correspondingly reduced the adaptive changes in cardiovascular design in the isolated SHR as judged by left ventricular weight and the hemodynamic characteristics of the resistance vessels in terms of the steepness of the resistance curve and the maximal pressor response

Discussion

Attempts to induce experimental neurogenic hypertension have frequently been made by exposing animals to prolonged environmental stress or to conflict situations (*e g* Hudak and Buckley 1961 Henry Meehan and Stephens 1967 Herd *et al* 1969) where an intact peripheral sympathetic nervous system seems to play a crucial role (Smookler *et al* 1973) This was also clearly demonstrated when rats for longer periods were intermittently stimulated with implanted electrodes in the integrating centre of such neurohormonal pressor responses *i e* the hypothalamic defence area (Folkow and Rubinstein 1966) In the long run frequent pressor increases will by enhancing the average burden on heart and vessels gradually initiate structural adaptations of the left ventricle systemic arteries and precapillary resistance vessels which when once established imply the establishment of a more persistent hypertension (*cf* Folkow *et al* 1973 1974)

According to previous findings (Hallback and Folkow 1974) the Okamoto type of spontaneously hypertensive rat exhibits a central hyperreactivity with respect to cardiovascular responses to alerting stimuli which is obviously hereditary in nature since it is present already in early prehypertensive phases These responses are in SHR not

pronounced concerning peak effects compared with NCR but they also seem to be more long lasting and elicited at a lowered threshold of alerting stimuli Accordingly neurogenic pressor responses of central origin are likely to occur more often in SHR than in NCR in their normal environment leading to about the same situation as if normotensive animals were exposed to frequent stressful episodes for long periods

Curiously enough the elimination of environmental influences may sometimes tend to exaggerate hypertension as has been described concerning inbred sound overloaded hypertensive female Wistar rats when sound deprived (Locett and Marwood 1973) However when SHR are raised in an environment of light deprivation this seems to retard the development of their hypertension (Brody Lais and Bhatnagar 1973) The aim of the present study was to eliminate the perhaps most common source of arousal *i e* the competition with other animals for food social rank and territory by means of isolation On the other hand the very procedure of isolation has in many cases led to accentuated aggressive behaviour amongst small rodents once confronted with social contacts as studied in rats by the mouse killing reaction (Yen Strangler and Millman 1959 Valizelli and Garattini 1972) However such rats may well be aggressive during confrontation but isolation by itself implies a considerable reduction in frequency and extent of arousal and alertness The isolated SHR in the present experiments (SHR_I) were not exposed to any type of somatomotor behavioural studies However according to the experienced keeper s observation they were as a group more easily handled during daily care and seemed to be less aggressive in their contacts with man than ordinary SHR which behaved in a much more aggressive way than did NCR Furthermore both SHR_I and NCR_I had significantly higher total body weights than their controls which rather speaks against the presence of the isolation syndrome as described by Hatch *et al* (1965) However once placed in the stimulation box for eliciting defence reactions SHR_I seemed to be somewhat more restless than SHR_C and differed still more from NCR in this respect

The cardiovascular responses to alerting stimuli imply however a more relevant parameter to investigate concerning the problem of hypertension than the somatomotoric behavioural responses to such stimuli since the former responses directly reflect the impact of environmental stimuli on the neurohormonal links governing the cardiovascular system Therefore the present experiments were designed to test the intensity and general character of the neurogenic cardiovascular responses to graded alerting stimuli The results indicate that the period of social deprivation did not lead to any reduction in the intensity of the exaggerated cardiovascular responses to standardized stress stimulations present in SHR If anything SHR_I responded more powerfully than the unisolated SHR_C However elimination of normal environmental stimuli such as social confrontations is likely to reduce the frequency of alertness episodes and the accordingly induced neurogenic pressor responses Thus if the enhanced prolonged and more frequent neurogenic cardiovascular responses to stress occurring in SHR really play an important role for the gradual initiation of their hypertension social deprivation would be expected to reduce the development of *the hypertensive state while exposure to chronic stress would have the opposite effect* The results of the present study clearly point in this direction since the progression and degree of hypertension in SHR_I was less pronounced than in SHR_C

The degree of structural adaptation of the precapillary resistance vessels in SHR which by its mere presence tends to raise flow resistance has been shown to be essentially a regional response to the level of blood pressure increase Thus when protecting only the hindquarter vascular bed of SHR from the hypertensive pressure level (Folkow *et al* 1971) or generally keeping pressure low by hypotensive drug treatment (Weiss Lundgren and Folkow 1974) from young age no hypertensive change in resistance vessel design occurs On the other hand when normotensive rats were made hypertensive by means of renal artery obstruction a nearly complete structural cardiovascular adaptation to high pressure occurs in about 3 weeks (Lundgren *et al* 1974) In agreement with such results the present investigation illustrates how the degree of structural adaptation of the left ventricle and resistance vessels was significantly less pronounced in SHR_I than in SHR_C and in close balance to the difference in mean arterial pressure between SHR_I and SHR_C

The subject of much debate in both SHR and man has been whether and then in which way changes in neurogenic activity contribute to initiate and maintain primary hypertension (*cf* Joint WHO symp on Pathogenesis of essential hypertension 1960 or The nervous system in arterial hypertension 1975) No doubt reduction of sympathetic influences in SHR from early age will greatly suppress the progression of hypertension (Clark 1971 Folkow *et al* 1972) and limits accordingly the vascular adaptive changes which seem to be the main element behind the chronically raised resistance (Folkow *et al* 1973 1974) This was also the case although to a less pronounced extent when the present SHR_I by means of their social isolation were exposed to less frequent central autonomic excitations even though their inherent cardiovascular hyperreactivity to alerting stimuli was fully maintained This clearly implies that sympathetic cardiovascular influences really contribute to the initiation of hypertension in SHR though preferentially by means of more or less transient excitations evoked by *e g* environmental stimuli as conveyed *via* the hypothalamic defence area, rather than in the form of sustained increases of sympathetic tone controlled at the bulbar level

However this central hyperreactivity to alerting stimuli implies not only the involvement of neurogenic cardiovascular mechanisms but also a spectrum of hormonal changes since the defence reaction also involves adjustments of the sympatho-adrenal, ACTH-corticoid and TSH tyroid systems which in fact, exhibit signs of hyperreactivity in SHR (Okamoto 1969 Tabei *et al* 1972) A hypothalamic hyperreactivity with frequent inductions of defence reactions will no doubt result in repeated though transient pressure increases so that the *average* pressure load on heart and precapillary vessels is somewhat raised and thereby initiate the abovementioned structural adaptations In addition, the various hormonal adjustments might directly or indirectly reinforce the interaction between neurogenic and structural mechanisms Once a structural adaptation of the precapillary resistance vessels is present any excitatory influence neural or hormonal will be potentiated in its hemodynamic impact so that the development of hypertension is further accelerated by establishing a vicious circle arrangement

This investigation was supported by grants from the Swedish Medical Research Council (B74-14X 16-10C) the Swedish National Association against Heart and Chest Disease and the Medical Faculty University of Göteborg AB Hässle generously covered part of the expenses for a technician Skilful technical assistance was offered by Miss Gunnel Andersson

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From the Institute of Zoophysiology University of Uppsala, Sweden

Distribution of an Adenohypophysial Constituent in the Body

II Quantitative Tissue Distribution in the Rat

By

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Received 18 September 1974

Abstract

HALL, H E LAKOMAA and J E KIHLSTRÖM *Distribution of an adenohypophysial constituent in the body II Quantitative tissue distribution in the rat* Acta physiol scand 1975 *93* 466–471

A peptide with a molecular weight of about 5 000 has previously been shown to affect the output of semen in frogs and probably also in mammals This sperm releasing substance is not part of any known gonadotropic hormone The tissue distribution of this substance has been investigated The iodinated substance has been injected into rats and the radioactivity content of the different tissues has been determined Iodinated rat albumin has been injected into other rats to determine the content of blood in the different tissues A formula has been derived to obtain a figure for real accumulation using the radioactivity content of the blood and of the tissues after injection of the sperm releasing substance and albumin respectively The sperm-releasing substance is incorporated into the adenohypophysis neurohypophysis, liver kidney lung, ovary uterus and some male sexual organs The causes for this distribution are discussed

A constituent from the adenohypophysis with a molecular weight of about 5 000 has been highly purified (Lakomaa 1974 b) This constituent induces the release of spermatozoa in frogs (Kihlstrom and Danninge 1970) Pituitaries from frogs perch rabbits cats cattle (Kihlstrom *et al* 1971) domestic fowls and humans (Lakomaa and Kihlstrom 1972) contain this constituent A crude adenohypophysial extract containing the sperm releasing substance (a name suggested by Lakomaa 1974 a) increase the output of semen in rabbits guinea pigs and mice (Lakomaa and Kihlstrom 1972) The sperm releasing substance is not identical with any known pituitary hormone (Lakomaa 1974 a and b) In the present study the sperm releasing substance has been iodinated and the distribution in the rat investigated

Material and Methods

The sperm releasing substance was prepared from bovine adenohypophysial acetone powder (Biofac A/S Copenhagen Denmark) according to Kihlström *et al* (1971) and Lakomaa (1974 b) with the exception of the final isoelectric focusing. The iodination of both the sperm-releasing substance and rat albumin (Fraction V Sigma) with Na I (carrier free Amersham, England and Studsvik Sweden) was carried out

TABLE I Radioactivity content in blood after injection of the iodinated compounds

Compound	Sex	Time	n	cpm/mg ± S D
Sperm-releasing substance	Males	1 min	10	211 ± 48
Sperm releasing substance	Males	3 min	20	170 ± 56
Sperm releasing substance	Females	1 min	10	309 ± 68
Sperm releasing substance	Females	3 min	10	233 ± 77
Albumin	Males	1 min	10	97 ± 110
Albumin	Females	1 min	10	439 ± 140

according to Greenwood *et al* (1963) 20 μg of the sperm releasing substance and albumin respectively were iodinated with 1 mCi each time

The rats used in this study were adult males (weighing 330–450 g) and adult nonpregnant females (weighing 0–310 g) of the Sprague Dawley strain (Anticimex Sollentuna, Sweden)

The preparation of the tissue samples was conducted according to Sjöholm and Ryden (1969) and Ågmo (1974) The rats were anaesthetized with 25 urethan given intraperitoneally (0 5 ml/100 g body weight) Thin polyethylene tubes were inserted into the right jugular vein and into the left common carotid artery The rats were heparinized with 100 IU of Heparin (Vitrum Sweden) and given 27–40 μCi of the iodinated sperm releasing substance in 0 3–0 5 ml phosphate buffered saline containing 0 1 M EDTA and 0 00 NaN_3 or 25–35 μCi of the iodinated rat albumin in 0 3 ml phosphate buffered saline with EDTA and NaN_3 through the jugular vein Injection of the sample took about 10 s After 1 or 3 min a sample of about 0 3 ml blood was collected from the carotid artery and immediately thereafter the animal was killed by exsanguination All the tissue samples to be studied were rapidly dissected out and carefully freed from connective tissue The samples were then washed in isotonic saline to remove any blood that may have adhered to their surfaces They were carefully dried on a filtering paper put in pre weighed polyethylene tubes and weighed to the nearest tenth of a milligram The tissues in the tubes were dis integrated with 1 ml 30 KOH to obtain equal geometry in the gammacounter The radioactivity content of the samples was counted in an Autowell II automatic gammacounter with an efficiency of about 10 and a background of 25–30 cpm

The radioactivity was determined per weight of the sample of each tissue inclusive blood (cpm/mg wet weight) The radioactivity content in the blood (Table I) varied considerably between different animals depending on the activity of the injected iodinated compound the weight of the rat and of the incubation time Therefore the best way to express the radioactivity content of the tissues and organs is in relation to the blood activity of each animal The means and standard deviations of the relative figures for the different tissues can be seen in Table II Values exceeding 300 of the expected values have been omitted as it can be suspected that the corresponding samples have been contaminated with external radioactivity Some animals were used in a special study of the epididymis (Table III) where the three parts caput corpus and cauda epididymis were dissected out from the same animals and compared with each other in regard to radioactivity content

Different tissues hold different amounts of blood containing non accumulated iodinated sperm releasing substance in order to eliminate these errors the distribution of blood plasma was studied using iodinated rat albumin

The real accumulation is thus the activity of the sperm releasing substance in each tissue excluding that of blood in the vessels of the tissue related to the blood activity This can be written and calculated from the following formula

Real accumulation = (Rel – Rel_a) (1 – Rel)

Derivation of the formula

Rel_s Activity in the tissue of sperm releasing substance related to the blood activity M n f ll m l (Table II)

Rel Dito of rat albumin

Act_{tx} Activity of sperm releasing substance in the whole tissue (cpm/mg)

TABLE II Relative activity of the tissues compared with that of blood Mean ± S D

Tissue	Injected iodinated compound					
	Sperm releasing substance				Albumin	
	Male 1 min n = 10	Female 1 min n = 10	Male 3 min n = 20	Female 3 min n = 10	Male 1 min n = 10	Female 1 min n = 10
Brain	0 040 ± 0 029	0 0?9 ± 0 009	0 048 ± 0 044	0 032 ± 0 012	0 0 0 ± 0 003	0 021 ± 0 00
Adenohypophysis	0 41 ± 0 06[a]	0 41 ± 0 11	0 40 ± 0 09	0 48 ± 0 16	0 17 ± 0 02	0 18 ± 0 04
Neurohypophysis	0 67 ± 0 19[a]	0 51 ± 0 12	0 54 ± 0 16[b]	0 80 ± 0 45	0 ?9 ± 0 14	0 25 ± 0 12
Hypothalamus	0 041 ± 0 010	0 033 ± 0 009	0 036 ± 0 013[b]	0 039 ± 0 011	0 024 ± 0 004	0 0 5 ± 0 012
Muscle	0 0?3 ± 0 007	0 023 ± 0 003	0 037 ± 0 014[b]	0 055 ± 0 032	0 012 ± 0 002	0 015 ± 0 007
Liver	0 23 ± 0 04	0 22 ± 0 04	0 32 ± 0 05	0 30 ± 0 08	0 13 ± 0 01	0 13 ± 0 02
Kidney	1 22 ± 0 62	1 37 ± 0 40	2 94 ± 1 60	2 52 ± 2 46	0 20 ± 0 07	0 18 ± 0 60
Lung	0 63 ± 0 07	1 29 ± 0 06	0 40 ± 0 11	0 36 ± 0 09	0.24 ± 0 09	0 22 ± 0 05
Testis	0 011 ± 0 003	—	0 016 ± 0 005	—	0 013 ± 0 005	—
Cauda epididymidis	0 016 ± 0 005	—	0 0?6 ± 0 014	—	0 011 ± 0 003	—
Vas deferens	0 053 ± 0 023	—	0 08? ± 0 027	—	0 021 ± 0 006	—
Seminal vesicle	0 061 ± 0 028	—	0 095 ± 0 023	—	0 0?3 ± 0 010	—
Coagulatory gland	0 048 ± 0 011	—	0 077 ± 0 024	—	0 018 ± 0 005	—
Prostate	0 060 ± 0 015	—	0 094 ± 0 026	—	0 022 ± 0 005	—
Ovary	—	0 23 ± 0 03	—	0 32 ± 0 07	—	0 10 ± 0 03
Uterus	—	0 075 ± 0 031	—	0 16 ± 0 07	—	0 0 6 ± 0 00?

[a] n = 9 [b] n = 18 [c] n = 19

Act_{tbs} Activity of sperm releasing substance in the blood of the tissue (cpm/mg)
Act_b Activity of sperm releasing substance in the blood Mean of all animals (cpm/mg Table I)
Rel_a shows the amount of blood in the tissue in fraction of the whole tissue
$1 - Rel_a$ thus represents the tissue without blood in the vessels

$Act_{tb} = Rel \; Act_{bs}$
$Act_{ts} = Rel_s \; Act_b$ (N B Act_{ts} is not necessarily the same as the mean of the activity in all animals)
$Act_{ts} - Act_{tbs}$ shows the activity in the tissue excluding that in the blood in the tissue but as this figure is dependent on the activity in the blood it is preferable to standardize it to blood activity i e to divide with Act_{bs} Thus

$$(Act_{ts} - Act_{tbs}) \, (Act_{bs}) = (Rel \; Act_b - Rel_a \; Act_b) \, (Act_{bs}) = Rel - Rel_a$$

shows the accumulation per mg tissue Since different tissues contain different amounts of blood it is preferable to show the accumulation per mg tissue excluding blood in the vessels Thus

Real accumulation $(Rel_s - Rel_a)/(1 - Rel_a)$

The standard deviation of the ratios in Table IV have been calculated according to Dahlberg (1948)

Results

The distribution of the sperm releasing substance and its accumulation into the different tissues can be seen from the tables and figures

High real accumulation occurs in the following tissues adeno- and neurohypophysis liver kidney lung, ovary and uterus The real accumulation in the other organs is very low (Fig 1 and 2) In parts of the male reproductive system there is some real accumulation giv

TABLE III Activity (relative that of blood) and real accumulation in three different parts of the epididymis Mean ± S D

	SRS 3 min n = 10	Albumin 1 min n = 5	Real accumul
Caput epididymidis	0 038 ± 0 007	0 013 ± 0 003	0 026
Corpus epididymidis	0 055 ± 0 01	0 019 ± 0 003	0 036
Cauda epididymidis	0 0 1 ± 0 007	0 012 ± 0 00[illegible]	0 008

ing similar values in all of these particular tissues The accumulation is however much lower than in the ovary In the epididymis the real accumulation is low but is greater in the caput and corpus epididymidis than in the cauda when comparing only the animals from which all three parts were taken (Table III)

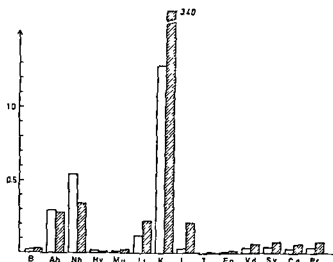
Discussion

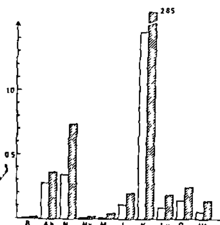
As some peptides (*e g* oxytocin and vasopressin) from the pituitary will be degraded very rapidly in the body the times when the distribution was studied were preset to only 1 and 3 min Since iodinated tyrosine residues derived from the degraded sperm releasing sub stance can be rebuilt into new peptides and proteins it could be suspected that incorporation in the tissues after longer intervals after injection need not necessarily be the result of an incorporation of the sperm releasing substance itself These short intervals after injection may however render the accumulation in the different organs incomplete and difficult to measure but until the biological half life of the sperm releasing substance has been determined the distribution after more than three minutes can hardly be studied

In most organs and especially in the organs rich in blood there is a low real accumulation

TABLE IV Ratio between relati e activity in the tissues compared to that of blood after 3 and 1 min Mean ± S D

	Male	Female
Brain	1 ± 1 4	1 1 ± 0 4
Adenohypophysis	1 0 ± 0 3	1 2 ± 0 5
Neurohypophys s	0 8 ± 0 3	1 6 ± 1 0
Hypothalamus	0 9 ± 0 4	1 2 ± 0 5
Muscle	1 6 ± 0 8	[illegible]4 ± 1 5
Liver	1 4 ± 0 3	1 4 ± 0 4
Kidney	2 4 ± 1 8	1 9 ± 1 9
Lung	1 5 ± 0 6	1 3 ± 0 4
Testis	1 4 ± 0 7	—
Cauda epididymidis	1 7 ± 1 0	—
Vas deferens	1 5 ± 0 8	—
Sem nal ves cle	1 6 ± 0 8	—
Coagulatory gland	1 6 ± 0 6	—
Prostate	1 6 ± 0 6	—
Ovary	—	1 4 ± 0 4
Uterus	—	2 [illegible] ± 1 3





Figs 1 2 Real accumulation (cpm/mg) (calculated with the formula derived in the text) in different tissues of the male (Fig 1) and female (Fig 2) Open bars incubation time 1 min Dark bars incubation time 3 min Br Brain Te = Testis A h = Adenohypophysis Ep = Cauda epididymidis N h = Neurohypophysis V d = Vas deferens Hy = Hypothalamus S v Seminal vesicl Mu = Muscle C g = Coagulatory gland Li Liver Pr = Prostate Ki = Kidney Ov = Ovary Lu = Lung. Ut = Uterus

of the iodinated sperm releasing substance (Fig 1 and 2) This suggests a common non specific accumulation presumably due to the much smaller size of the sperm releasing substance compared to albumin (molecular weights 5 000 and 68 000 respectively) This smaller size makes it possible that the iodinated sperm releasing substance can leave the plasma and enter the interstitial fluid The rate of accumulation is also approximately the same in most organs with an approximately 50% higher amount of accumulated compound after 3 min than after one (Table IV)

The high accumulation in the kidneys may be due to a rapid excretion of the sperm releasing substance as has been described earlier (Kihlstrom and Hall 1974) In both sexes there is an accumulation in the hypophysis This can at least in the posterior part be the result of an incorporation of free radioactive iodine which accumulates in the neurohypophysis (Jentzer 1953) As the sperm releasing substance is derived from the adenohypophysis it is probable that the accumulation in this part of the pituitary is caused by a reuptake of the substance

Since the known functions of the substance are localized to the genital organs it is not surprising to find accumulation in these organs of both sexes most apparent in the ovary but radioactivity is also found in vas deferens seminal vesicle coagulating gland and prostate Lakomaa (1974 a) observed a depletion of the ovarian ascorbic acid content in pseudopregnant rats after injection of partially purified material containing the sperm releasing substance which can explain the high accumulation in the ovary The incorporation in the three parts of the epididymis was much lower as might have been expected from the autoradiographical studies (Kihlstrom and Hall 1974) but the relation between the parts is the same as was found earlier

A comparison between the autoradiographical studies (Kihlstrom and Hall 1974) and these quantitative studies must be made In the autoradiographical pictures an uptake in the epididymis was apparent The sperm releasing substance was not incorporated into any other organ to the same extent as could be seen from the pictures However the other male genital organs with the exception of the testes were somewhat difficult to find in the sections The substance could thus be incorporated there to some extent but an incorporation as high as in the epididymis should easily have been discovered

This work has been supported by grants from the Helge Ax on Johnson Foundation Långmanska kultur fonden and Hierta Retzius Foundation and from the Swedish Natural Sc ence Research Council to Jan Erik Kihlström The skilled technical assistance of Miss Eva Britt Norlin is gratefully acknowledged

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Acta physiol scand 1975 93 472–476
From the Department of Physiology I Karolinska Institutet Stockholm Sweden

Duration of Central Action of Angiotensin II Estimated by its Interaction with CSF Na^+

By

B ANDERSSON L G LEKSELL and M RUNDGREN

Received 24 September 1974

Abstract

ANDERSSON B L G LEKSELL and M RUNDGREN *Duration of central action of angiotensin II estimated by its interaction with CSF* Na^+ Acta physiol scand 1975 93 472–476

In pre hydrated goats an urge to drink persisted for approximately half an hour after combined infusions of angiotensin II and hypertonic (0 5 M) NaCl into the lateral or third cerebral ventricle The intraventricular infusion of angiotensin/glucose solution having no dipsogenic action of its own markedly accentuated the dipsogenic and antidiuretic effects of the subsequent intraventricular infusion of hypertonic NaCl The possibility is discussed that angiotensin may be bound at periventricular receptor sites where it continues to interact with Na in eliciting thirst and ADH release for about half an hour

Previous studies in the goat have revealed that the dipsogenic and antidiuretic hormone (ADH) releasing effects of angiotensin II infused into the third cerebral ventricle are dependent on the [Na] of the cerebrospinal fluid (CSF) This has led to the suggestion that angiotensin in some manner facilitates the stimulation of periventricular Na^+ receptors which primarily are influenced by the CSF [Na] (Andersson 1971) In those studies it was observed that combined intraventricular infusions of angiotensin and hypertonic NaCl solution had much greater dipsogenic and ADH releasing effects than either stimulus alone whereas similar infusions of the octapeptide dissolved in solutions of non-electrolytes were on the whole ineffective The intraventricular infusions of angiotensin together with hypertonic NaCl invariably elicited thirst also in pre hydrated animals On some occasions these pre hydrated goats were not allowed free access to water It was then observed that the urge to drink persisted for a considerable length of time after the infusions In contrast no obvious dipsogenic after-effect was seen on the few occasions when infusions of merely hypertonic NaCl elicited thirst in pre hydrated animals without free access to water Since these preliminary observations indicated a relatively long lasting central action of angiotensin II administered into the third ventricle they have provided the incitement for the experiments reported here

Methods

Animals 6 adult female goats (b.wt. 35±2 kg) were used. The animals were routinely confined in metabolism cages, and all experiments were conducted in these cages with no extra restraint on the goats.

Ventricular implantations and infusion technique The goats were supplied with permanent cannulae in the anterior part of the third ventricle (2 animals), and in the lateral ventricle near the foramen of Monro (4 animals). The three-cannula system used, and the infusion technique have been described in detail previously (Åkerlund, Andersson and Olsson 1973). In all experiments CSF was observed to drain out of the permanent cannula on gentle compression of the neck immediately before, and after the infusions. Thus, free mixing of the infused solutions with the CSF occurred in all experiments. The rate of infusion was always 10 μl/min.

Hydration and test for the urge to drink All experiments were made in hydrated animals which had received, by stomach tube, 100 ml/kg of 38° C water 80 min before the intraventricular infusions were started. At the start of the infusions a water diuresis was fully developed. In the beginning of each experiment the goats, as usual, had free access to drinking water placed in front of them. However, when an intraventricular infusion induced drinking the goats were allowed to take only about 100 ml of water. Then the water bucket was covered with a lid. Towards the end of the infusion period, and at intervals thereafter, the lid was temporarily removed to test the persistance of the urge to drink. On each of these trials the goats were allowed to take only negligible amounts of water, and the trials were repeated until the animals no longer showed any interest in water.

Angiotensin Angiotensin II (Hypertensin, Ciba) was infused into the CSF at a dosage of 0.4 ng/kg min. The octapeptide was either dissolved in 0.5 M NaCl or 0.3 M d-glucose solutions.

Urine samples and analyses Urine was collected in 10 min samples via a retention catheter inserted into the urinary bladder. An Advanced Instruments Inc. osmometer was used for determinations of urine and plasma osmolality. The mean pre-infusion plasma osmolality was 290 mosm/kg in the hydrated animals, and this value was used for calculations of the renal free water clearance (C_{H_2O}).

Results

1) *Combined angiotensin/NaCl infusions*

Five 30 min infusions (10 μl/min) of angiotensin II (0.4 ng/kg min) dissolved in 0.5 M NaCl solution were made into the *third ventricle* during hydration and water restriction. All infusions elicited thirst after 2 to 4 min. The urge to drink remained throughout the infusions and on the average for 27 min (range 24 to 30 min) after cessation of the infusions. 3 out of 5 control infusions of merely the 0.5 M NaCl solution also elicited an urge to drink, but the animals lost their interest in water within 5 min after cessation of the saline infusions.

Similar 30 min infusions were made during hydration and water restriction in 3 of the goats having cannulas implanted into the *lateral ventricle*. All combined infusions (no. 13) elicited thirst after a latency time of 5 to 9 min. The mean dipsogenic after-effect was 29 min (range 23 to 34 min). 4 out of 9 control infusions of merely 0.5 M NaCl also elicited an urge to drink in these animals. However, the goats took no notice of water offered to them 5 min after the infusions were stopped.

2) *Alternating angiotensin and NaCl infusions*

Since the dipsogenic after-effect of the combined infusions indicated a long lasting central action of angiotensin, it was of interest to find out whether a remaining action of the octapeptide would strengthen the dipsogenic and ADH releasing effects of a subsequent inf
of hypertonic NaCl in the hydrated animals.

Angiotensin II (0.4 ng/kg min) dissolved in isotonic glucose solution was

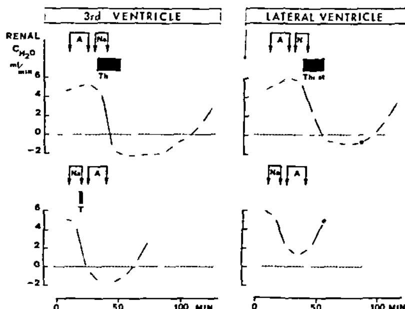


Fig. 1 Strengthening of the dipsogenic and antidiuretic effects of intraventricular infusions of hypertonic (0 5 M) NaCl solutions (*Na*) by the preceding (in itself ineffective) intraventricular infusion of angiotensin II dissolved in 0 3 M d glucose solution (*A*) in 2 hydrated goats The responses to equivalent infusions of hypertonic saline made before the angiotensin/glucose infusions are shown below The animals which did not have free access to water exhibited obvious urge to drink during the periods (*black*) marked "THIRST" and "T" Dosage of angiotensin II = 0 4 ng/kg min Rate of infusion = 10 µl/min C_{H_2O} = free water clearance

3 animals third ventricle one animal) for 15 or 25 min periods ending 5 min prior to the start of a 10 min infusion of 0 5 M NaCl The reverse sequence of infusions was used as a control in the 6 experimental series performed

The infusion of angiotensin/glucose in itself did not elicit any visible urge to drink, and had no or very little antidiuretic effect. All the subsequent infusions of hypertonic saline elicited thirst and the urge to drink outlasted the infusion periods by 5 to 14 min (mean 8 min) Renal C_{H_2O} turned negative in the first post infusion period of urine collection and remained negative for 40 to 80 min (Fig. 1 above)

The effects of hypertonic NaCl infusions performed prior to the angiotensin/glucose infusions were much less pronounced Thirst became apparent towards the end of 2 out of 6 such infusions but on both occasions the urge to drink had disappeared before the subsequent angiotensin/glucose infusion was started Also the antidiuretic effect of the NaCl infusions was considerable less accentuated when they preceded the angiotensin/glucose infusions (Fig. 1 below)

Discussion

Hypertensive dipsogenic and ADH releasing effects are exponents of a central angiotensin action, the physiological importance of which largely remains to be elucidated (*cf* Severs and Daniels-Severs 1973) A striking sodium angiotensin interaction demonstrated by intraventricular infusions in the goat (*cf* Andersson *et al* 1972) indicates that sodium ions

of the CSF and angiotensin influence the central control of fluid balance by acting synergistically on the same central receptor mechanism(s) Whether to discuss these so far imaginary receptors in terms of sensors primarily activated by sodium or by angiotensin remains an academic question until more is known about the phenomenon However as a working hypothesis it has been suggested (Andersson 1971) that angiotensin promotes the stimulation of periventricular Na^+ sensitive receptors either (a) by facilitating transependymal Na^+ movement (b) by sensitizing the receptors for the existing CSF [Na^+] or (c) by facilitating the passage of Na^+ into receptor cells Clear evidence in support of the idea that periventricular Na^+ receptors participate in the regulation of water intake and the release of ADH and that the stimulation of these receptors is facilitated by angiotensin has recently been provided It has been shown that intraventricular infusions of iso- or hypertonic solutions of saccharides which in all probability reduce the CSF [Na^+] inhibit the dipsogenic antidiuretic and natriuretic effects of intracarotid infusions of hypertonic NaCl solutions in the goat (Olsson 1973) Furthermore Olsson and Kolmodin (1974) have found that infusions of angiotensin II into the CSF of the lateral ventricle (in a dosage having negligible antidiuretic and no dipsogenic effect of its own) markedly potentiate the ADH releasing and thirst eliciting effects of simultaneous intracarotid infusions of hypertonic NaCl in hydrated goats The present results are in accordance with these observations and show in addition that potentiation also may be obtained when the infusions of angiotensin and hypertonic saline are not concurrent Intraventricular angiotensin/glucose infusions which were ineffective *per se* markedly strengthened the dipsogenic and antidiuretic effects of a subsequent elevation of the CSF [Na^+] This sodium angiotensin interaction remained apparent for 20 min or longer after the angiotensin/glucose infusions were discontinued (Fig 1 persistent urge to drink)

Hardly any enzymatic degradation of angiotensin II seems to take place in CSF (Mouw *et al* 1971) Therefore the octapeptide may remain active in this fluid for a considerable time after its infusion into the cerebral ventricular system It could provide a straightforward explanation for the persistent action of angiotensin observed in this study However the CSF bulk flow of approximately 0 2 ml/min in the goat (Pappenheimer *et al* 1962) must be expected to carry away fairly rapidly angiotensin distributed in the small volume of CSF present in the anterior part of the third ventricle This appears to be the site at which an elevated CSF [Na^+] elicits drinking in the goat (Andersson Dallman and Olsson 1969) and consequently the site where angiotensin must be expected to interact with Na^+ in inducing drinking The time delay for passage through the anterior part of the third ventricle of angiotensin originally distributed in the CSF of the lateral ventricle must have been somewhat longer Nevertheless this did not show up as any obvious difference in the dipsogenic after-effect of combined angiotensin/NaCl infusions in the lateral and third ventricles It provides further indirect evidence against the possibility that the observed prolonged central action of angiotensin was due to the presence of free angiotensin in the CSF after the infusions It rather indicates that angiotensin II may be bound at periventricular receptor sites where the octapeptide continues to interact in some manner with Na^+ in eliciting thirst and ADH release for approximately half an hour (perhaps by maintaining a high [illegible] [Na^+] in primarily sodium sensitive receptors)

Studies performed by Volicer and Loew (1971) provide some excuse for this very speculative attempt to explain the long lasting action of angiotensin II infused into the cerebral ventricular system of the goat. By radioautographic examination they have shown that ^{14}C-angiotensin II injected intravenously in mice enters the brain via the blood-CSF barrier to be found around the third ventricle and the aqueduct.

This work was supported by the Swedish Medical Research Council (Project 04X 503) The generous gift of Hypertensin from the Hässle–Ciba–Geigy Company is gratefully acknowledged.

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Acta physiol scand 1975 93 477–482
From the Department of Physiology University of Lund Sweden

Influence of Autonomic Nerves and Drugs on Myoepithelial Cells in Parotid Glands of Cat

By

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Received 28 September 1974

Abstract

THULIN A *Influence of autonomic nerves and drugs on myoepithelial cells in parotid glands of cat* Acta physiol scand 1975 *93* 477–482

When a single electrical shock is applied to either the parasympathetic or to the sympathetic nerves a pressure rise is seen in the parotid duct of the cat probably due to myoepithelial cell activation No secretion occurred on single impulse stimulation When repetitive stimulation was used both divisions of the autonomic nervous system could evoke secretion The motor response to sympathetic stimulation was mediated via α receptors while secretion mainly was mediated via β receptors

Key words Salivary gland myoepithelial cell, autonomic nerves

Myoepithelial cells have been demonstrated around acini and along intercalary ducts in the parotid gland of the cat (Garrett 1966 a) Morphological investigation after sympathetic and parasympathetic denervation suggest that both divisions of the autonomic nervous system are in close relationship with myoepithelial cells (Garrett 1966 b and 1972) From physiological experiments Stromblad (1955) discussed the possibility that adrenaline activates contractile elements in the parotid gland of the cat Emmelin *et al* (1968) found that stimulation of the parasympathetic nerves with single shocks elevated the parotid duct pressure without evoking any secretion and they proposed that this pressure effect was exerted by activation of myoepithelial cells

In the present investigation the influence of the autonomic nerves and drugs on the myoepithelial cells was further investigated

Methods

3 cats weighing 1 8–3 6 kg were used. They were anesthetized with chloralose 80 mg/kg, given through a cannula in a femoral vein after induction with ether A tracheal tube was inserted The right or the left parotid duct was exposed and cannulated with a glass or a polyethylene cannula which was connected either with a displacement transducer or with a glass capillary using a technique earlier described (Thulin 1974). This made it possible to study pressure changes to different stimuli in a closed duct system and salivary flow in an open duct system. In the closed system the pressure was raised to 70 mm H

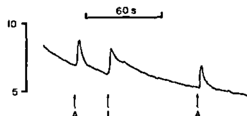


Fig 1 Pressure rises to stimulation of the auriculotemporal nerve using single shocks (A) and to a fluid injection (I) in the closed duct system Calibration in mm Hg

before each stimulation 0 007 µl was the smallest volume displacement which evoked a detectable pressure rise in the closed duct system In the open system a fluid displacement was recorded by watching the meniscus in the glass capillary through a dissecting microscope at × 10 magnification A volume displacement of 0 015 µl could be detected with this method

In 10 expts the auriculo-temporal nerve was exposed as described by Burgen (1964) and in 17 expts the cervical sympathetic trunk was exposed The nerves were stimulated with supramaximal strength (4–8 V) and with a duration of 2 ms using a bipolar electrode and a Grass S4 stimulator The following drugs were injected i v acetylcholine methacholine atropine adrenaline phenylephrine isoprenaline dihydroergotamine propranolol and D-( )-N isopropyl p nitrophenylethanolamine (INPEA)

Results

Parasympathetic nerve stimulation

A single shock applied to the auriculo temporal nerve always evoked a pressure rise in the closed duct system (Fig 1) the latency between the application of the stimulus and the pressure response was less than one second and the maximal pressure rise was 3 7 mm Hg ± 0 7 (mean ± S E) in 9 expts Repetitive stimulation 0 1–1 0 Hz was needed to evoke secretion in the open duct system A stimulation frequency which evoked secretion sometimes elicited a biphasic pressure rise in the closed duct an early quick rise and a second more gradual pressure elevation Atropine 0 1 mg/kg abolished the flow responses and the pressure changes seen to parasympathetic stimulation

Injection of physiological saline solution (0 1 µl) in the closed duct system at a suitable speed imitated the pressure response to a single shock applied to the auriculo temporal nerve (Fig 1) However the pressure returned more slowly to the initial level than after an auriculo-temporal stimulus In the open system this fluid injection could easily be detected as secretion

Parasympathomimetic drugs

Acetylcholine and methacholine evoked pressure rises in doses which did not evoke secretion (Table I) Fig 2 shows the pressure responses to subsecretory doses of acetylcholine

TABLE I Threshold doses (µg kg) for pressure rise and secretion Mean values ± S E n = number of experiments

	n	Pressure	Secretion
Acetylcholine	7	0 009 ± 0 001	0 19 ± 0 056
Methacholine	5	0 006 ± 0 00?	0 09 ± 0 0 9

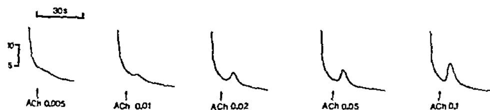


Fig. 2 Pressure responses to different subsecretory doses of acetylcholine (ACh) Doses in μg/kg

No separate second pressure rise could be detected when secretory doses of the parasympathomimetic drugs were injected

Sympathetic nerve stimulation

The secretory threshold to sympathetic nerve stimulation was 0 1–5 0 Hz in 16 expts In 1 expt, sympathetic stimulation 20 Hz did not evoke secretion although the pupil dilated widely When stimulation frequencies of 10–20 Hz were used the fluid movement in the glass capillary started 10–20 s after the application of the stimulus Within 1 min the flow almost ceased but after about 2 min secretion started again and lasted as long as the stimulation was continued When the nerve stimulation was finished the flow of saliva usually accelerated during 10–30 s During the following 2–5 min the meniscus in the glass capillary moved backwards although it did not reach the initial level Dihydroergotamine 0 5–1 0 mg/kg abolished the initial fluid movement while the total amount of secretion was very little diminished No secretory response to sympathetic stimulation was seen after propranolol 1–3 mg/kg had been given

In the closed system a single shock applied to the sympathetic trunk usually evoked a pressure rise (Fig 3) This pressure elevation started 2–4 s after the application of the stimulus and the maximal size of the response was 0 5 mm Hg With repetitive stimulations which evoked secretion the initial pressure rise became steeper and larger and was usually followed by a later small and gradual pressure rise This second pressure rise was accentuated after the stimulation was finished The early pressure rise was abolished by dihydroergotamine 0 5–1 5 mg/kg while the second pressure elevation was abolished by β blocking drugs

Sympathomimetic drugs

The secretory threshold to isoprenaline was 0 5–10 μg/kg in 4 expts In the closed duct system isoprenaline evoked a late and gradual pressure rise when secretory doses were given Propranolol 1–3 mg/kg, abolished the secretory and pressure responses to isoprenaline

In 5 expts the secretory threshold to phenylephrine varied between 10–100 μg/kg, while in 2 experiments 200 μg/kg did not evoke secretion Secretion started 30–60 s after the administration of the drug and it was greatly diminished or abolished by β blocking drugs In the closed duct system subsecretory doses of phenylephrine usually evoked a quick pressure rise which was abolished by dihydroergotamine 0 5–1 0 mg/kg (Fig 4) Secretory doses of phenylephrine evoked a more longlasting pressure elevation which was shortened after β-blocking agents had been given

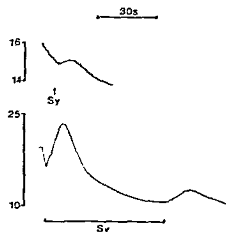


Fig. 3 The upper record shows the pressure response to a single shock applied to the sympathetic trunk. The lower record shows the pressure response on sympathetic stimulation at 20 Hz.

The secretory threshold to adrenaline was 1 0–20 μg/kg in 7 expts in 1 expt 20 μg/kg did not evoke secretion. Secretion to adrenaline was almost abolished by β-blocking drugs. In the closed duct system secretory doses of adrenaline evoked a late and gradual pressure rise This pressure response was abolished by propranolol or INPEA In 3 of 8 expts an early steep pressure rise was seen to subsecretory doses of adrenaline This was followed by the above described late pressure elevation when the secretory dose was reached Dihydroergotamine 0.5 mg/kg, abolished this early pressure response to adrenaline

Discussion

Several earlier investigators (see Langley 1898 and Babkin 1950 Ekstrom and Emmelin 1974 a and b) have shown that the auriculotemporal nerve is the principal secretory nerve to the cat parotid gland. The observation in this study that a single shock applied to the auriculo-temporal nerve does not evoke secretion is in accordance with earlier experiments by Emmelin *et al* (1968) Fritz and Botelho (1969) also found that a single shock applied to the parasympathetic nerve did not evoke secretion nor did it change the cell membrane potential in the cat parotid gland After a fluid injection into the parotid duct the pressure declined more slowly than after the parasympathetic nerve stimulus (Fig. 1) which contrasts to the findings in the submandibular gland of the cat by Darke and Smaje (1971) Similar to

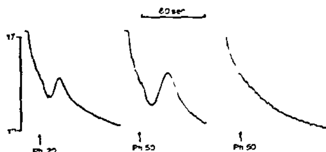


Fig. 4 Pressure responses to phenylephrine (Ph) 20 μg/kg and to phenylephrine 50 μg/kg before and after dihydroergotamine 1 mg/kg.

earlier investigations in dog (Emmelin *et al* 1969) subsecretory doses of acetylcholine and methacholine evoked a pressure rise in the cat parotid gland It seems therefore reason able to suggest that the pressure response seen to a single shock applied to the auriculo temporal nerve and to subsecretory doses of parasympathomimetic drugs is due to activation of myoepithelial cells

Stromblad (1955) Ohlin and Stromblad (1958) Nordenfelt (1965) Ekstrom and Em melin (1974 b) have shown that stimulation of the sympathetic trunk evokes a small and varying amount of secretion from the cat parotid gland which was also found in the present experiments The initial fluid movement seen in the open duct system corresponds to the initial pressure rise in the closed duct and is probably due to myoepithelial cell activation mediated via α receptors A motor response due to activation of α receptors could also be elicited when adrenaline and phenylephrine were given

The secretory response to sympathetic nerve stimulation and to sympathomimetic drugs seems mainly to be caused by activation of β receptors in the clo ed duct it corresponds to the late more gradual pressure rise The secretion usually accelerated during a short time after sympathetic stimulation and this was recorded as an accentuation of the late pressure rise in the closed system (Fig 3)

Batzri *et al* (1972) have shown that a β adrenergic effect can be exerted by phenylephrine in rat parotid slices since it releases endogenous catecholamines This might explain the secretory effect of phenylephrine in the present experiments which could be abolished by β blocking agents

This work was supported by grants from the Medical Faculty of Lund Sweden

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Acta physiol scand. 1975 93 483–489

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The Effect of Stimulation of the Cervical Sympathetic Chain on Regional Cerebral Blood Flow in Monkeys

A Study with Radioactively Labelled Microspheres

By

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Received 9 October 1974

Abstract

ALM A *The effect of stimulation of the cervical sympathetic chain on regional cerebral blood flow in monkeys A study with radioactively labelled microspheres* Acta physiol scand 1975 93 483–489

15 μm labelled microspheres were used to determine the effect of stimulation of the cervical sympathetic chain on blood flow through different parts of the brain and through some extracerebral tissues of the head in monkeys. Unilateral stimulation at 10 Hz for one min caused mean reductions in blood flow through the masseter muscle the parotid gland and the tongue of 76–94 percent in 9 monkeys, while in the same animals there were no convincing effects on regional cerebral blood flow

In a previous report radioactively labelled microspheres were used to determine the effect of stimulation of the cervical sympathetic chain on cerebral and ocular blood flow in cats (Alm and Bill 1973 a) In the brain regional cerebral blood flow (rCBF) was determined for grey and white matter respectively of the frontal temporal and occipital lobes Sympathetic stimulation did not result in any statistically significant reduction in rCBF in either normocapnic or hypercapnic animals with the possible exception of white matter of the frontal lobe in normocapnic cats

James Millar and Purves (1969) reported a reduction of about 30 percent for blood flow through both grey and white matter in the baboon as the result of stimulation of the cervical sympathetic chain The possibility of a species difference made it important ... microsphere method in a primate The following results were obtained in monkeys As in cats ocular blood flow was also determined The effect stimulation on blood flow through the various tissues of the eye will be

Methods

9 cynomolgus monkeys (*Macaca irus*) of both sexes weighing between ? 3 and 3 1 kg were used Anesthesia was induced with sodium methohexital 50–100 mg i m (Brietal Sodium®, Lilly) and maintained by i v injections of pentobarbital sodium (Penthotalsodium®, Abbott) The femoral artery and vein on one side were cannulated with polyethylene catheters the vein for injections and the artery for measurements of the air damped mean arterial blood pressure (MAP) with a pressure transducer (EMT 35 Elema Schönander Solna Sweden) The femoral artery on the other side was cannulated for blood sampling during and after the injection of the microspheres All animals were tracheotomized and artificially ventilated by a Palmer pump Arterial P_{O} P_{CO} and pH were determined with a Beckman Physiological Gas Analyzer model 160 Heparin 2 500 I U was given i v to prevent clotting The animals were placed on a heating pad The body temperature determined with a rectal thermometer was between 36 0 and 39 0 C

Both cervical sympathetic chains were cut and the distal end on one side was stimulated with a bipolar platinum electrode interelectrode distance 1 mm connected to a stimulator AEL model 111 (American Electronic Laboratories) The electrode was placed below the superior cervical sympathetic ganglion and the area was flooded with mineral oil to prevent drying of the nerve Square wave pulses of 1 ms duration were used at 10 Hz The stimulation intensity was adjusted to give maximal pupillary response usually 6–8 V Stimulation was started 1 min before the blood flow determination

The labelled microsphe e method

The method as used in this laboratory has been described in detail previously (Alm and Bill 197) 0 ml of a 5 percent suspension of 15±5 μm microspheres in saline was injected over ‿0 s into the left ventri le of the heart The microspheres (3 M Company St Paul Minnesota) were labelled with [14] Yb and the specific activity was 1 mCi/g. From the start of the injection and for the following 2 min blood was collected from a cannulated femoral artery in 70 s samples to obtain a reference flow At the end of the 1 0 s period the animals were killed with i v KCl and dissected Blood and tissue samples were weighed and the radioactivity was determined by gammaspectrometry The blood flow through each tissue sample was calculated according to the formula

$$\text{Blood flow} = \frac{\text{activity in tissue sample} \times \text{reference blood flow}}{\text{activity in reference blood}}$$

where blood flow is expressed in mg/min and activity in cpm

As a rule double samples were taken bilaterally from grey and white matter of the frontal and the occipital lobe from pia parotid gland masseter muscle and tongue Samples were also taken from cardiac muscle and from various abdominal organs Both lungs were removed weighed and minced and the radioactivity of 4 representative samples was determined to estimate the overall leakage of microspheres through the systemic ascular bed

Results

At the time of injection of the microspheres the following data were obtained MAP 151 ±7 cm H_2O (n = 9) arterial P_{O} P_{CO} and pH 88 4 ± 2 5 mm Hg (n = 8) 37 7 ± 1 7 mm Hg (n = 8) and 7 54 ± 0 02 units (n = 8) respectively

The calculated blood flow for the lung was 48 g/min per 100 g tissue

Table I presents the flow values for the control side and the percent reduction observed on the stimulated side for cerebral tissues and for parotid gland masseter muscle and tongue On the control side blood flow through grey matter is significantly lower for the frontal than for the occipital lobe (p < 0 01) The calculations are based on paired data The ratio (flow through grey matter)/(flow through white matter) was 1 8 ± 0 2 (p < 0 005) and 2 6 ± 0 2 (p < 0 001) for frontal and occipital lobes respectively The difference between the ratios for the two lobes 0 8 ± 0 2 was significant (p < 0 01 paired data)

TABLE I B ood flow through sympathectomized side percent reduction of blood flow on stimulated side and significance levels (Student s t test paired data) Mean±S E 9 animals

Tissue	Blood flow (g/min per 100 g tissue)	Percent reduction	Significance level
Grey matter			
Frontal lobe	43± 4	0 7±5 3	
Occipital lobe	69± 6	17.2±5 8	p<0 0_5
White matter			
Frontal lobe	24± .	3 4±5.5	
Occip tal lobe	76± 2	6 3±4 5	
Pia	109±19	−0 9±7 6	
Parotid gland	49± 9	9_ ±4	p<0 001
Masseter muscle	1 4± 6	76 ±3	p<0 001
Tongue	56±10	94 ±5	p<0 001

Highly significant blood flow reductions were found in all extracerebral tissues studied, while among the cerebral tissues only grey matter of the occipital lobe showed a possible significant reduction

Table II presents the flow values for cardiac muscle and for various abdominal organs

Discussion

Blood flow through control side

Since one aim of the present study was to determine blood flow through various small parts of the eye including the optic nerve head it was necessary to use a large dose of microspheres in order to minimize the statistical errors Such a large dose may disturb the circulation and in the present study there was an increase in MAP of 10–20 cm H_2O as a result of the injection of the spheres In a previous study (Alm and Bill 1973 b) two series of monkeys were presented one where the same dose as used in the present study was injected and one where the dose was about 10 percent of this dose Flow values through the cardiac

TABLE II Blood flow through various tissues Mean±S E (n)=number of an mals

Tissue	Blood flow (g m n per 100 g tissue)
Card ac muscle	291±39 (9)
Liver (hepatic artery)	42± 6 (9)
Spleen	185±35 (9)
Adrenal gland	350±80 (9)
Kidney cortex	18±.. (9)
Small intestine	46± 8 (9)
Uterus	46±14 (5)
Ovary	81±3 (5)

for the posterior Against this possible explanation speak 1) the absence of effect on white matter of the occipital lobe in the present study James *et al* (1969) found a reduction for white matter of the same order as for grey matter 2) The absence of a significant effect in the experiments in dogs by Meyer and Klassen (1973) They injected two batches of differently labelled particles one during stimulation and one after recovery and thus could compare both the two hemispheres during unilateral stimulation and one hemisphere with and without stimulation 3) The absence of such regional differences in the experiments in cats (Alm and Bill 1973 a) In fact the numerically largest reductions were seen in the frontal lobe supposed to be supplied with nerves from both sides

Thus with a stimulation technique that reduces blood flow through various extracerebral tissues by 76–94 percent there was no convincing reduction in rCBF

The author wishes to thank Professor Anders Bill for helpful suggestions during the experiments and the preparation of the manuscript, and Mrs Anita Östberg and Miss Monica Thorén for valuable technical assistance

This investigation was supported by a grant EY 00475 from the National Eye Institute Public Health Service U.S.A. and by a grant B74-14X 147 from the Swedish Medical Research Council

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Acta physiol scand 1975 93 490–499

From the Psychochemistry Institute Rigshospitalet Copenhagen Denmark

Blood–Brain Transfer of D-Glucose, L-Leucine, and L-Tryptophan in the Rat

By

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Received 14 October 1974

Abstract

BRENDER J P E ANDERSEN and O J RAFAELSEN *Blood–brain transfer of D glucose L leucine and L tryptophan in the rat* Acta physiol scand 1975 *93* 490–499

Recently Oldendorf developed a method for measurement of the early uptake of substances from the capillary bed into brain tissue after a single capillary passage of a bolus injected rapidly into the common carotid artery of the rat The uptake of a test substance is expressed relative to the uptake of a highly diffusible reference substance tritiated water In this study experimental data are presented allowing to correct the uptake values for the unknown loss of tritiated water so that the fractional unidirectional uptake (Extraction E) can be calculated This method is used to investigate the uptake kinetics for D glucose L leucine and L tryptophan For the three substances investigated the uptake kinetics involved both saturation and linear kinetics K_m values of 11 mM for D glucose 0 16 mM for L leucine and 0 19 mM for L tryptophan were found The uptake capacity V_m was calculated using a blood flow value of 0 85 ml (g min) V_m was for D glucose 1 6 for L leucine 0 027 and for L tryptophan 0 024 μmol/(g × min) The D glucose V_m in the present study is comparable with V_m values in the literature and indicates that the method may be employed for quantitative analyses of the blood brain transfer of solutes

Key words blood–brain barrier capillary permeability cerebral blood flow D glucose L leucine L tryptophan uptake kinetics

The purpose of this study was to investigate the uptake kinetics of D glucose L leucine and L tryptophan from blood to brain tissue The results for the brain are compared with the results obtained for extracerebral tissues where transport occurs by simple diffusion across the capillary wall

The method described by Oldendorf (1970 and 1971) to examine the uptake of various substances into the rat brain offers advantages compared with the indicator-dilution technique (Chinard *et al* 1955 and Crone 1963) of being simple fast adaptable to small animals and applicable to simultaneous measurements of the uptake in other tissues also mainly supplied with blood from the common carotid artery

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Method

Animals Male rats of the Wistar strain weighing 270–330 g have been used. The animals were fasted 18 to 4 h before the experiments with free access to tap water

Anesthesia 480 µl of a 5% solution of pentobarbital sodium (Nembutal®) injected intraperitoneally

Isotopes Uniformly labelled ^{14}C-D glucose ^{14}C-D fructose ^{14}C-sucrose ^{14}C-L leucine and ^{14}C-L tryptophan from the Radiochemical Centre Amersham, England and tritiated water from the Atomic Energy Research Establishment, Risø Denmark

Injection solution 200 µl Krebs-Ringer phosphate buffer[1] (pH = 7 40–7 45) containing µCi ^{3}HOH and 0.5 µCi ^{14}C labelled test substance The concentration of D-glucose was 5 6 mM except in the experiments with sucrose and with D-fructose and in the glucose saturation experiments.

Procedure During anesthesia the right common carotid artery was exposed and placed on an elevator (Doppel Elevatorium, PHAU Germany) The rat was placed in a guillotine a hypodermic needle (internal diameter 0 0 mm, external diameter 0 40 mm) was inserted into the common carotid artery and 200 µl of the isotope solution was injected the injection was performed rapidly (ca 0 5 s) to minimize mixture with the blood The needle was left in place and the animal was decapitated 15 s after the injection except when otherwise indicated The duration of the procedure from the midline incision was 3 min or less.

Tissue preparation and counting The tissues examined were sampled from the side of the head ipsilateral to the injection site

The cerebral hemisphere was quickly dissected free and the basal ganglia were removed with a curved pair of tweezers. The cortex was divided into 3 parts of about equal size As no difference was found between the different parts of the cortex the parietal part was chosen as representative for the brain cortex

The tongue tissue from the apex to the papillae vallatae was sampled by cutting. In some of the animals the temporal and the masseter muscles, the bony mandibular angle and the exorbital lacrimal gland were also used. The preparation of tissue lasted less than 3 min after the decapitation

The samples were subjected to routine digestion and preparation for liquid scintillation counting 2 ml per sample of equal parts of dioxan and of Soluene 100 (Packard) were used as a solubilizer Equal parts of a non-radioactive brain digested in the same way and with aliquots of the original injection solution were used as standards of reference After digestion for 15 h at 50 C, 450 µl 35% H_2O_2 was added for bleaching, followed 1 h later by 500 µl 1 M HCl and again 1/2 h later by 15 ml liquid scintillator (Instagel, Packard)

The bone tissue samples were digested in 1 ml 1 M HCl overnight, and then the digestion was continued 4 hours after addition of 1 ml Soluene-dioxan solution Finally 15 ml of Instagel was added

The samples were counted in a Packard Tri-Carb 33 0 Liquid Scintillation Counter Quench corrections were made according to Hendler (1964)

Calculations The Uptake Index was calculated as (Oldendorf 1970 and 1971)

$$UI = \frac{^{14}C_{tissue} / ^{3}H_{tissue}}{C_{inj.sol.} \quad H_{inj.sol.}} \quad (1)$$

The average regional cerebral blood flow $\bar{f}$ was determined using a formula developed for highly diffusible substances by Ingvar and Lassen (196)

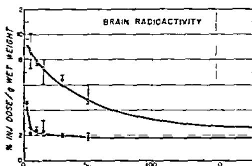
$$\bar{f} = \lambda \frac{0.693}{T_{1/2}} \, ml/(g \times min) \quad ( )$$

where λ is the blood–brain partition coefficient (1 04), and $T_{1/2}$ is the duration (in minutes) for the tissue content to decrease to one half during the initial part of the desaturation. The formula is applicable to blood flow measurements under certain assumptions, *i.e.*, that the tissue is homogeneous, that there is complete mixing within the extracellular space and no arterio-venous shunting, and that the flow is equal for all capillaries of the tissue examined, resulting in a mono-exponential curve.

Transport kinetic calculations were undertaken for the transport from blood into brain tissue according to:

$$v = \frac{V_m}{1 + K_m/s} + k \, s, \quad (3)$$

[1] The BUI(15) for 5 6 mM D-glucose in this buffer (31) showed no difference compared with Ringer bicarbonate buffer (31) and Ringer HEPES buffer (31%) used by Oldendorf (HEPES: N-hydroxyethylpiperazine N -ethanesulphonic acid). Neither in experiments with L-tryptophan was any significant difference found between the buffers.



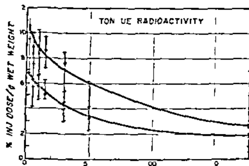


Fig 1 Fig 2

Fig. 1 Brain tissue content (per cent of injected dose/g brain wet weight) of ^3H (from ^3HOH) and ^{14}C (from ^{14}C-D-glucose) as a function of time after common carotid artery injection ^3H (O) and ^{14}C (●) Each point represents the median of 5–11 animals (except the 180 s point with only 3 animals) with the interquartile interval given as vertical bars

Fig. 2 Tongue tissue content (per cent of injected dose/g tongue wet weight) of ^3H (from ^3HOH) and ^{14}C (from ^{14}C–D glucose) as a function of time after common carotid artery injection ^3H (O) and ^{14}C (●) Each point represents the median of 5–11 animals (except the 180 s point with only 3 animals) with the interquartile interval given as vertical bars

where v_t is the transport velocity for the substance (the UI (corrected for the reference substance non-ideality) multiplied by s and flow rate) s the concentration of the test substance in the injectate and k a constant. K_m and V_m are the transport characteristics normally used for saturable systems conforming to Michaëlis-Menten kinetics, and k s is a correction factor assumed to be linear which may partly be free diffusion (or a transport mechanism conforming to diffusion kinetics) and partly intravascular residue This linear component of the equation is referred to as diffusion in the text.

Statistics Non parametric statistics (Mann Whitney U test) were used throughout the study (Siegel 1959) Every figure represents the median with the interquartile interval given in parentheses (Snedecor 1956)

Results

Fig. 1 shows the isotope content of the brain as a function of time after the injection with an initial uptake of ^3HOH of about 10% and a subsequent continuous decrease representing the wash out From a semilogarithmic plot of these values the average cerebral cortical blood flow was calculated to 0 85 ml/(g min) The initial uptake of ^3H was similar for brain and tongue whereas the initial ^{14}C content (from radioglucose) of the tongue (Fig. 2) was much greater than that of brain From Fig 2 is also seen that the ^{14}C content of the tongue was washed out proportionally to that of ^3H while in brain (Fig. 1) the ^{14}C content was nearly constant between 5 and 180 s Assuming that the decrease of ^{14}C between 2 and 5 s (Fig. 1) represents the wash out from the vessels of ^{14}C not taken up the brain retains nearly all the test substances once taken up within the period of investigation

The Brain Uptake Index at 15 s BUI(15) was used to calculate the Extraction E defined as the fractional uptake of the test molecules by taking into account the *fractional retention* of tritiated water at 15 s R(15)

$$E = BUI(15) \quad R(15) \tag{4}$$

R(15) was assessed in the following way 1) As shown by Bolwig and Lassen (1975) in normocapnic rats only about 50% of the H activity reaching the brain is retained initially that

TABLE I UI(15) (per cent) in different tissues for D glucose (5 6 mM) L tryptophan (0 08 mM 1 leucine (0 0 mM)

Tissue	D-glucose		L tryptophan		L leucine	
Brain	31 (8–35)	n = 8	30 (27–31)	n = 6	22 (19–.6)	n = 5
Apex of tongue	64 (56–68)	n = 8	65 (63–71)	n = 8	66 (63–70)	n = 5
Exorbital lacrimal gland	65 (56–75)	n = 8	—		—	
Mandibular angle	70 (57–96)	n = 4	70 (60–75)	n = 7	—	
Masseter muscle	77 (66–8?)	n = 7	64 (61–83)	n = 6	—	
Temporal muscle	78 (73–83)	n = 5	75 (59–97)	n = 6	—	

Each figure represents the median with the interquartile interval given in parentheses

is at time zero 2) of the initially retained ^3H activity about 80 % is still present in the brain after 15 s (Fig 1) Hence R(15) is taken to be 0 50 0 80 ≃ 0 40

The BUI(15) was 31 for 5 6 mM D glucose 30 for 0 08 mM L tryptophan and 22% for 0 20 mM L-leucine (Table I) In the other tissues investigated muscle glandular epithelium, and bone tissue the Uptake Indices were much higher about 65–75% There were only small variations from tissue to tissue with the substances investigated Table II shows that the BUI(15) of D glucose was different from that of sucrose and D fructose whereas for the tongue the Uptake Indices were similar for the three substances investigated Also in the exorbital lacrimal gland similar results were obtained for D-glucose and sucrose

The extraction E was used to calculate the transport rate v_s of the test molecules

$$v_s = E \quad \bar{f} \quad s \tag{5}$$

where $\bar{f}$ is the average cerebral blood flow of normocapnic nembutal anesthetized rats (0 85 ml/(g × min)) Equation (3) expresses that the uptake (the unidirectional flux) is assumed to involve both a saturable and a linear uptake With this method the transfer kinetics can be illustrated by means of the fractional uptake multiplied by the substrate concentration in the injection solution v (fractional transfer relative to HOH)

$$v = UI(15) \quad s \tag{6}$$

which means that $v = v_s/(R(15) \quad \bar{f})$

Fig. 3 illustrates the plot of v versus s for the uptake of D glucose and D-fructose into tongue and brain (for the tongue the results were similar with the two substances) and Fig 4 illustrates the uptake of L leucine and L-tryptophan into the brain and the tongue Hyperbolic curves indicate saturability while linear curves through origin (constant fract uptake for all concentrations) indicate diffusional uptake

TABLE II UI(15) (per cent) of D glucose su rose and D fructose (all 5 6 mM) in different tissues

Test substance	Brain	Tongue	Exorbital lacrimal gl	n
D glucose	31 (28–35)	64 (56–68)	65 (56–75)	8
Sucrose	1 7 (1 6–2 2)	56 (53–65)	57 (46–73)	1.
D fructose	2 0 (1 8–2 6)	69 (66–74)	—	13

Each figure represents the median with the interquartile interval given in parentheses

Determination of V_m and K_m implies elimination of the k s component in equation (3) As for the K_m value the determination does not imply the constants R(15) and f indicating that one need not go beyond the simple v – s plot The elimination of k s was done graphically from the slopes of the curves at the highest concentrations giving v (v – v – k s/(R(15) × $\bar{f}$)) The glucose transfer has not reached complete saturation indicating that the k s component is slightly overestimated by graphical elimination however this was of the same size 2 0 % BUI values as the D fructose BUI(15) of 2 2 % in accordance with Crones suggestion (1965 b) of D fructose as an indicator of the component of non facilitated transfer The linear component of the uptake kinetics for the two amino acids as obtained from the slope of the curves at the highest concentrations were slightly larger than that for D glucose (probably due to the lipophilic side chains) 3 6 % BUI values for L leucine and 3 3 % for L tryptophan

As a reciprocal plot for the K_m determination the Hanes plot s/v versus s (Segal 1959)

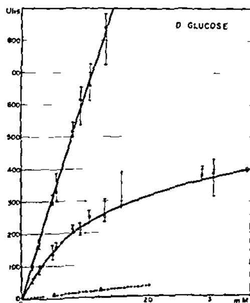


Fig 3 v (– UI(15) s) for the sugars D glucose and D fructose as a function of concentration of test substance in the injection solution Brain D glucose (O) tongue D glucose (●) brain D fructose (▲) Each point represents the median of 3–5 animals with the interquartile interval given as vertical bars

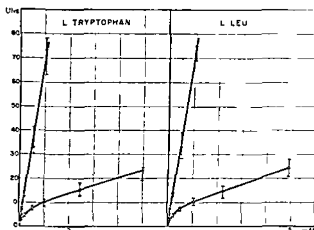


Fig. 4 v (=UI(15) × s) for the amino acids L-tryptophan and L leucine as a function of concentration of test substance in the injection solution Brain (O) and tongue (●) Each point represents the median of 3–5 animals with the interquartile interval given as vertical bars

was for statistical reasons chosen in preference to the analogous Lineweaver Burk plot giving the best visual fit of the estimated points From the Hanes plots in Fig 5 and 6 K_m values of 11 mM for D glucose 0 19 mM for L tryptophan and 0 16 mM for L leucine were obtained V_m values were calculated from equation (3) giving values of 1 6 μmol/(g × min) for D-glucose 0 024 μmol/(g × min) for L tryptophan and 0 027 μmol/(g min) for L-leucine

Discussion

Oldendorf found a BUI(15) of 22 5 % for 5 mM D glucose which is somewhat lower than the 31 % found here at 5 6 mM D glucose a divergence not solely due to the difference in glucose concentration an explanation of this lower value of Oldendorf's might be a somewhat higher cerebral blood flow than in the present study as indicated by the ³H wash out presented by Oldendorf (1970) The calculation for the D glucose by means of equation (4) gave an extraction of 12 % at a blood flow of 0 85 ml/(g min) this is considerably lower than the initial extraction of 25% found by Crone (1965 b) and others but equals the net steady state extraction of about 10 With this method it is not possible to obtain extraction versus time curves in one animal to obtain initial extractions this means that the results correspond to an overall extraction Correction for the amount of ³HOH bypassing the brain in a single capillary passage—instead of being exchanged with brain extracellular water—has been made according to experimental data obtained in this group indicating a HOH bypassing as high as 50% in normocapnic rats corresponding to an initial extraction of about 50 (Bolwig and Lassen 1975) In other species the initial extraction of water has been found to be nearly complete (72–96%, Yudilevich and De Rose 1971) in a single capillary passage Species differences (e g flow rate of the dog compared to the rat) and differences in experimental procedure (e g type and duration of anesthesia) may account for this discrepancy

The fractional uptake of D glucose relative to ³HOH in the tongue was independ-

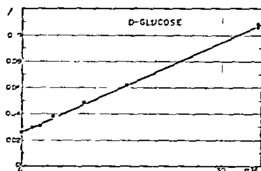


Fig. 5 s/v versus s plot for D-glucose (v' = v corrected for the concentration proportional uptake, see text) Apparent K_m = 11 mM

time Together with the rapid wash out of [14]C from the tissue this indicates that UI values in the tongue mainly represent escape of the substances across the capillary wall and to a far lesser degree cellular uptake The assumption that the results might primarily be a function of the exchange across the capillary wall is supported by the experiment with the extra cellular tracer sucrose sucrose and D-glucose having similar permeability coefficients

After correction for the diffusion and the intravascular residue of the test substance a satisfactory fitting of our results to Michaelis-Menten kinetics (Fig. 5 and 6) was obtained. Several investigators have demonstrated the saturability of the D-glucose and the amino acid transport into brain (Baños *et al* 1970 Growdon *et al* 1972 LeFevre and Peters 1966 Neame 1968 and Yudilevich *et al* 1972) and this was confirmed in the present study For D-glucose transfer Bachelard found K_m values of 5 mM in guinea pig brain *in vitro* (1971) and 7 mM in rat *in vivo* (1972) Growdon *et al* a K_m of 6 mM in mice *in vivo* (1971) and Buschiazzo *et al* a K_m of 7 mM in nephrectomized rats (1970) From Crone s findings (1965 b) a K_m of about 2 mM can be calculated for dogs *in vivo* Pappenheimer and Setchell (1973) found K_m values of 5 5 mM and 6 mM respectively for the sheep and rabbit *in vivo* Oldendorf reported a K_m of 20 mM in the rat *in vivo* (1971) In the present study 11 mM was found.

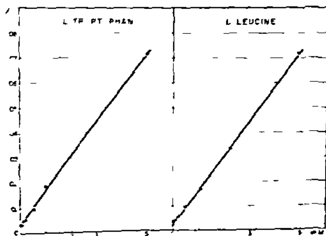


Fig. 6. s/v versus s plot for L tryptophan and L leucine (v' = v corrected for the concentration proportional uptake, see text) Apparent K_m = 0 19 mM for L tryptophan and 0 16 mM for L leucine.

We calculated a K_m of 0 19 mM for L tryptophan and 0 16 mM for L leucine the e l values are not directly comparable with those from *in vitro* experiments made with slice in the literature Previous *in vivo* experiments with amino acids (Banos *et al* 1970 and Old i dorf 1973) showed saturable transfer kinetic without indication of the kinetic para meters

As the cerebral blood flow of the rat is much higher than that of other experimental animals such as sheep rabbit and dog the problem of the flow rate introducing non linear elements into the interpretation of the kinetic data has importance The higher flow rate in the rat may thereby partly be responsible for the relatively higher apparent K_m value than those reported in the literature

A maximal transport capacity V_m of 1 6 μmol/(g × min) for the transport of D glucose into brain compares well with the previously published *in vivo* results of 1 5 μmol/(g × min) (Buschiazzo *et al* 1970) for nephrectomized rats 2 1 μmol/(g × min) (Growdon *et al* 1971) for mice 1 24 μmol/(g × min) (Bachelard) for rat and 2 6 and 2 8 μmol/(g min) respec tively for sheep and rabbit (Pappenheimer and Setchell 1973) Determination of V_m values with the present method is subjected to some uncertainties as the reliability of V_m in con trast to that of K_m depends on the behavior of the reference substance The bypassing of the reference substance implies a correction factor in the V_m determination In the present study the determination of V_m and the extraction of D glucose was made by means of a correction factor of 0 5 (Bolwig and Lassen 1974) omitting this correction a V_m of 3 1 μmol/(g × min) and an extraction of 24 % will be obtained both of which are the values to be found if the reference substance was ideal with respect to equilibration across the capillary wall

The blood flow determination with this method was based on the assumption that the wash out curve for the indicator represented a mono-exponential function The HOH wash-out curve for the brain in the semilogarithmic plot is linear only up to the point of 50 s hereby indicating that the flow value might be slightly overestimated However the present flow value is in agreement with other estimates in the literature Sapirstein and Hanusek (1958) found a cerebral blood flow of 0 82 ml/(g min) using iodoantipyrine and Goldman and Sapirstein (1973) found 0 88 ml/(g min) using the indicator fractionation technique and antipyrine as a diffusible indicator both studies in rats during pentobarbital anesthesia A cerebral cortical blood flow of 1 00 ml/(g min) for normocapnic rats have been found with a number of tracers using the Kety Schmidt method (Eklof *et al* 1974) indicating further that the antipyrine method may lead to erroneous results

In brain it is still questionable which transport processes are studied with the present method—as it is with other available methods (Chinard *et al* 1955 Crone 1965 a and 1971 Crone and Thompson 1973) The net uptake of substances from the vascular bed during a single capillary passage in brain may be a function of the transport across the capillary wall the distribution into extracellular space and the transport into nervous tissue or glial cells The present tissue sampling method and the indicator dilution method are comple mentary to one another and both may add to the knowledge of the blood–brain barrier The findings of Brightman and Reese (1970) have confirmed that the endothelial cells brain capillaries represent a diffusion limitation but the possible existence of serial

to the diffusion of molecules from blood to brain represents a problem which still awaits its final solution

The research was undertaken while two of us were holding student research scholarships from the University of Copenhagen (J B and P E A) The research was supported by grants from P Carl Petersens Fond and the Danish Medical Research Council

We express our appreciation to Dr N A Lassen Department of Clinical Physiology Bispebjerg Hospital Copenhagen for valuable discussions

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Acta physiol scand 1975 93 500–504
From the Department of Pharmacology School of Medicine Linkoping Sweden

Effect of Alloxan-diabetes on the Metabolism of Rabbit Colon Smooth Muscle

By

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Received 14 October 1974

Abstract

ARNQVIST H J *Effect of alloxan diabetes on the metabolism of rabbit colon smooth muscle* Acta physiol scand 1975 *93* 500–504

The metabolism of colon smooth muscle from normal and alloxan diabetic rabbits was studied *in vitro* Glucose uptake incorporation of glucose ^{14}C into glycogen and incorporation of leucine ^{14}C into protein were determined These three parameters were all depressed in diabetic smooth muscle incubated in a medium containing 5 6 mM glucose Raising the glucose concentration in the medium to 22 2 mM almost doubled the glucose uptake both in diabetic and normal smooth muscle and at this glucose concentration no significant difference in this parameter was found Insulin (0 1 U/ml) added *in vitro* stimulated glucose uptake incorporation of glucose ^{14}C into glycogen and incorporation of leucine ^{14}C into protein in diabetic colon smooth muscle Insulin stimulated glucose uptake to the same degree in normal and diabetic colon smooth muscle while its effect on glucose ^{14}C and leucine ^{14}C incorporation tended to be somewhat more *pronounced in diabetic colon muscle*

The metabolic activity of smooth muscle cells is probably of importance for the development of vascular diseases (Adams and Bayliss 1969 Wissler 1968 Wolinsky 1973) Since diabetes mellitus is associated with an increased frequency of disorders of the vascular system it is of interest to know how insulin and the diabetic state influence the metabolism of smooth muscle

In previous investigations (Arnqvist 1973 a 1974) the effect of insulin on smooth muscle metabolism was studied *in vitro* in bovine mesenteric arteries and rabbit colon smooth muscle Insulin stimulated glucose uptake glycogen synthesis amino acid transport and the incorporation of amino acid into protein in these preparations In comparison with the corresponding effects in rat skeletal muscle the effects of insulin on the smooth muscle were weaker and appeared later The effects on smooth muscle could only be demonstrated at insulin concentrations above the physiological range (Arnqvist 1971 1974)

Alloxan-diabetes has been reported to decrease the metabolism of glucose in rat and rabbit aorta (Wertheimer and Ben Tor 1962 Urrutia *et al* 1962 Mulcahy and Winegrad 1962) and the incorporation of amino acid into protein in rat aorta (Ben Tor and Wertheimer 1964)

In the present investigation the effect of diabetes on smooth muscle metabolism was studied in rabbit colon smooth muscle Glucose uptake glycogen synthesis and incorporation of amino acid into protein were determined and were found to be depressed in smooth muscle from alloxan-diabetic animals

Material and Methods

Animals Male rabbits weighing 2–3 kg were used In order to decrease the biological variation and make it possible to detect small differences in metabolism between normal and diabetic animals the rabbits were divided into pairs Each pair consisted of siblings of the same weight one of which was randomly chosen for alloxan treatment while the other served as a control Diabetes was induced by i v injection of alloxan monohydrate 1?5–150 mg/kg. Control animals were injected with saline The rabbits were considered diabetic if random blood glucose determinations 2 weeks after alloxan administration showed values greater than 300 mg/100 ml. No insulin treatment was given to the diabetic animals. Before the experiments the rabbits were starved for 20–24 h Both rabbits in each pair were sacrificed on the same day by a blow on the neck and colon smooth muscle was dissected out as previously described (Arnqvist 1973 b) The *in vitro* incubations of normal and diabetic smooth muscle were run in parallel.

Incubation technique The incubation procedure has been described in detail earlier (Arnqvist 1973 b) The tissue samples were incubated in 25 ml flasks containing 4 ml Krebs-Henseleit bicarbonate buffer and a gas phase of 95% O_2 and 5 CO For determination of glucose uptake 10 ml flasks containing 2 3 ml of the buffer were used When added the concentration of insulin (pork monocomponent insulin from Novo) in the medium was 0 1 U/ml After the incubation period the tissue samples to be analyzed were frozen at −80 C in Frigen ($CFCl_3$) containing solid CO_2

Analytical The analytical methods were the same as used previously (Arnqvist 1973 a, 1974) In short glucose was determined enzymatically by hexokinase and glucose 6-phosphate dehydrogenase (Slein 196) The incorporation of glucose C into glycogen was measured after digestion of the tissue with KOH and precipitation of glycogen by ethanol. For determination of incorporation of leucine C into protein the tissue samples were homogenized in trichloroacetic acid and the precipitated protein was purified according to Arvill and Ahren (1967) The radioactivity of the glycogen and protein was counted in a liquid scintillation detector

Statistics The significance of the effect was calculated from the differences between paired observations, using Students t test

Results

The glucose uptake in rabbit colon smooth muscle was determined after an incubation period of 180 min in a medium containing 5 6 mM glucose with and without added insulin (0 1 U/ml) From Fig. 1 A it is seen that the glucose uptake was significantly lower ($p < 0.001$) in smooth muscle from alloxan-diabetic rabbits than in that from normal rabbits Insulin moderately increased the glucose uptake in both normal and diabetic tissue (Fig 1 A Fig. 2) In the presence of insulin the glucose uptake was still significantly lower in the diabetic tissue

To test how variations of the glucose concentration in the medium influenced the glucose uptake of colon smooth muscle the glucose uptake was also determined at a glucose concentration of 22 2 mM in the incubation medium Fig. 3 shows that raising the glucose concentration from 5 6 to 22.2 mM markedly increased the glucose uptake both in the diabetic and normal colon smooth muscle At this glucose concentration no difference in glucose uptake was found between normal and diabetic smooth muscle

The incorporation of C labelled glucose into glycogen was depressed in colon smooth muscle from diabetic rabbits when measured after incubation for 180 min in 5

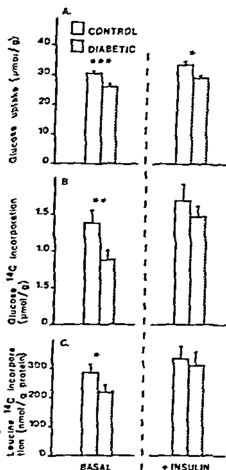


Fig. 1 Glucose uptake (A) incorporation of glucose ^{14}C into glycogen (B) and incorporation of leucine ^{14}C into protein (C) were determined in rabbit colon smooth muscle from normal and diabetic animals All tissue samples were incubated for 180 min in a medium containing 5 6 mM glucose with and without added insulin (0 1 U/ml) Tracer amounts of glucose ^{14}C were added when the glucose ^{14}C incorporation was measured 0.01 mM leucine ^{14}C was present in the incubation medium in the experiments on leucine ^{14}C incorporation The asterisks denote the significance of the differences between normal and diabetic colon muscle $= p < 0.05$ $= p < 0.01$ $= p$ 0 001 Vertical bars indicate one S E. (n = 17 in Fig. 1 A n = 17 in Fig. 1 B and C)

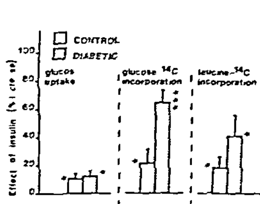


Fig. 2

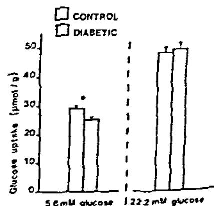


Fig. 3

Fig. 2. The effect of insulin on glucose uptake glucose ^{14}C incorporation and leucine ^{14}C incorporation in normal and diabetic colon muscle expressed in percent of the basal values. The figure is based on the data presented in Fig. 1 The asterisks denote the significance of the effect of insulin

Fig. 3 Effect of glucose concentration on glucose uptake in normal and diabetic rabbit colon smooth muscle Glucose uptake was determined after incubation for 180 min in 5 6 mM or 2 mM glucose. Vertical bars indicate one S E. Significance is denoted as in Fig. 1 (n = 1_)

glucose ^{14}C (Fig 1 B) Addition of insulin stimulated the glucose ^{14}C incorpor o muscle from both diabetic and control animals The effect of insulin was most p orou on the diabetic colon muscle (Fig. 2) In the presence of insulin the glucose ^{14}C incorpora i was not significantly lower in the diabetic muscle

The incorporation of leucine ^{14}C into protein in colon smooth muscle was determ ned after an incubation time of 180 min in a medium containing 5 6 mM glucose and 0 01 mM leucine ^{14}C From Fig. 1 C it is seen that the leucine ^{14}C incorporation was decreased in diabetic smooth muscle Insulin augmented the incorporation of leucine ^{14}C in diabetic as well as in normal colon smooth muscle (Fig. 2) and when insulin was present the leucine incorporation was not significantly depressed in diabetic muscle

Discussion

The results of this investigation indicate that the metabolism of rabbit colon smooth muscle is influenced by alloxan-diabetes Glucose uptake incorporation of glucose into glycogen and incorporation of leucine into protein were all decreased in diabetic smooth muscle Similar metabolic changes are caused by alloxan-diabetes in intima media preparations from rat and rabbit aorta (see introduction) of which smooth muscle is a major cellular constituent This suggests that diabetes affects the metabolism of vascular and intestinal smooth muscle

The glucose uptake in diabetic colon muscle was decreased in comparison with colon muscle from control animals when the glucose concentration of the incubation medium was 5 6 mM Raising the glucose concentration of the incubation medium from 5 6 to 22 2 mM almost doubled the glucose uptake in diabetic smooth muscle The glucose uptake was also augmented in normal smooth muscle in agreement with earlier observation (Arnqvist 1973 b) At the glucose concentration of 22.2 mM the glucose uptake did not differ significantly between normal and diabetic tissue The smooth muscle of the diabetic animal is exposed to an elevated blood glucose concentration *in vivo* and the glucose uptake of diabetic smooth muscle *in vivo* may rather be elevated than depressed

In alloxan-diabetic rat aorta glucose uptake is stimulated by insulin (Wertheimer and Ben Tor 1962) and in diabetic rabbit aorta the incorporation of glucose into glycogen and lipids is augmented by insulin (Mulcahy and Winegrad 1962) Mulcahy and Winegrad (1962) found no effect of insulin on glucose uptake in normal or diabetic rabbit aorta *in vitro* but the reduced glucose uptake in diabetic aorta could be restored by administration of insulin *in vivo* In this study insulin was found to stimulate glucose uptake incorporation of glucose into glycogen and incorporation of leucine into protein in diabetic and normal rabbit colon smooth muscle In the presence of insulin the glucose uptake was still slightly lower in diabetic than in normal colon muscle while the incorporation of glucose into glycogen and the incorporation of leucine into protein were not significantly depressed Expressed as percentual increase the effect of insulin on glucose uptake was of similar magnitude in normal and diabetic colon muscle (Fig. 2) while the effects on glucose and leucine incorpora tion tended to be relatively larger in the diabetic muscle This suggests that smooth muscle is at least as sensitive as normal colon smooth muscle to the

The fact that insulin counteracted the metabolic changes produced by alloxan-diabetes suggests that they were caused by insulin deficiency This interpretation must however be regarded with some caution as other hormones such as growth hormone and cortisone are known to be of importance for the metabolic changes in diabetes (Krahl 1961) Thus it cannot be excluded that the metabolic alterations in diabetic smooth muscle are secondary to the hormonal imbalance in the diabetic state and not a direct effect of insulin deficiency

The excellent technical assistance of Mrs Lena Burlin is gratefully acknowledged Financial support was given by Nordisk Insulinfond and by the Swedish Medical Research Council (14X 101)

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Acta physiol scand 1975 93 505–514
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The Influence of Acute Normovolemic Anemia on Cerebral Blood Flow and Oxygen Consumption of Anesthetized Rats

By

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Received 22 October 1974

Abstract

BORGSTROM L H JOHANNSSON and B K SIESJO *The influence of acute normovolemic anemia on cerebral blood flow and oxygen consumption of anesthetized rats* Acta physiol scand 1975 93 505–514

The influence of acute normovolemic anemia on cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR_{O_2}) was studied in normocapnic rats under nitrous oxide anaesthesia The arterial hemoglobin content was reduced to values of about 12, 9 6 and 3 g (100 ml)$^{-1}$ by arterial bleeding and substitution with equal volumes of homologous plasma The CBF increased in proportion to the reduction in hemoglobin content to reach values of 500–600 per cent of normal at extreme degrees of anemia but CMR_{O} remained unchanged Cerebral venous P_{O} and oxygen saturation did not decrease below normal values indicating that tissue hypoxia did not develop However since the increase in CBF at hemoglobin concentrations of below 9 g (100 ml)$^{-1}$ was far in excess of that expected from the decrease in viscosity the results indicate that dilatation of cerebral resistance vessels occurred This dilatation which was obviously related to the fall in arterial oxygen content cannot be explained by any of the current theories proposed to explain cerebral hyperemia in hypoxia

Although there is much information on cerebral blood flow (CBF) and cerebral oxygen consumption (CMR_{O}) in acute and chronic anemias important problems remain unsolved Chronic anemias in man (pernicious sickle cell and other types) were studied by Scheinberg (1951) and by Heyman *et al* (1952) who found an increased CBF and decreased CMR_{O} These authors concluded that cerebral hypoxia contributed to the increase in CBF and decrease in CMR_{O} However since administration of 85–100 per cent oxygen (Heyman *et al* 1952) or specific therapy (Scheinberg 1951) did not bring CMR_{O} back to normal it appeared doubtful if the fall in CMR_{O} was due to hypoxia alone

Subsequent experiments on acute anemia have been largely designed to study the factors responsible for the cerebral hyperemia Haggendal and Norbäck (1966 b) found little variation in CBF in dogs over a hematocrit range of 70–30 per cent and concluded that the increase in CBF occurring below a hematocrit of about 30 per cent was due to hypoxia, the decrease in viscosity playing only a minor role Data presented by of workers (Häggendal and Norback 1966 a) suggested that CMR was ma

hematocrit of 20 per cent Similar results were reported by Michenfelder and Theye (1969) who reduced hemoglobin concentration from 12 4 to 5 6 g $(100\ ml)^{-1}$ in the same species In their experiments CBF increased from 77 to 108 ml $(100\ g)^{-1}$ min^{-1} but CMR_O remained constant Since cerebral venous P_O was reduced from 37 to 30 mm Hg cerebral hypoxia may be assumed to have contributed to the increase in CBF In a subsequent study on man (Paulson *et al* 1973) the hematocrit was reduced from 38 to 28 per cent This procedure gave an increase in CBF by 15–20 per cent but since cerebral venous P_O remained unchanged the authors concluded that the hyperemia was largely due to reduced blood viscosity and that cerebral hypoxia should not have been present

The present experiments were designed to study circulation and oxygen supply to the rat brain in acute normovolemic anemia The blood hemoglobin concentration was reduced to minimal values of 3 g $(100\ ml)^{-1}$ *i e* to about 20 per cent of normal with subsequent *measurements of CBF CMR_O and cerebral venous P_O The objectives of the experiments* were to study the efficiency of the circulatory adaptation in moderate to severe anemia and to obtain further information on the cause of the hyperemia A preliminary communication on part of the present material has been published (Jóhannsson and Siesjo 1974 a)

Methods

Since the procedures and methods used in the present study were similar to those reported in two previous communications (Jóhannsson and Siesjö 1974 b Borgström *et al* 1974 a) only the main outline is given here with an emphasis on differences in procedures

The experiments were performed on unstarved male rats (350-400 g) that were anesthetized with ? per cent halothane tracheotomized and maintained artificially ventilated on 70 per cent N_2O and 30 per cent O_2 The respirator was set to give a Pa_{CO} of 35–40 mm Hg and body temperature was kept close to 37 C Both femoral arteries and one femoral vein were cannulated and the posterior part of the superior sagittal sinus was exposed for sampling of cerebral venous blood

Thirty min after the end of the operative procedures and when a respiratory steady state was at hand the blood hemoglobin concentration was reduced by gradual bleeding from one arterial catheter and gradual replacement with homologous plasma via the venous catheter Fresh plasma was obtained from donor rats that were anesthetized with halothane (2–2 5) Bleeding from the arterial catheter occurred at a rate of about 1 ml min^{-1} Infusion of plasma was done at the same rate and the amount of plasma given was equal to the loss of arterial blood In order to obtain blood hemoglobin concentrations of 9 and 3 g $(100\ ml)^{-1}$ about 5 and ?? ml respectively of whole blood had to be substituted with plasma In these groups the final hemoglobin concentrations were thus attained after about 5 and 0 min respecti ely

There were two main series of experiments In the first series (series A) the animals were allowed a steady state period of 30 min at blood hemoglobin concentrations of about 9 6 and 3 g $(100\ ml)^{-1}$ before CBF and CMR_O were measured A control group was obtained by maintaining animals at normal hemoglobin concentration for a comparable period This control group was identical to that previously reported (Jóhannsson and Siesjö 1974 b) CBF was measured according to the Kety and Schmidt principle (1948), using ^{133}Xenon and repeated sampling of arterial and cerebral venous blood during desaturation (Norberg and Siesjö 1974 see also Jóhansson and Siesjö 1974 b) The partition coefficient (λ) used was that given by Veal and Mallet (1965) corrected for hematocrit CMR_O was calculated by multiplying CBF with the arteriovenous difference in oxygen content (AVD_O) In each animal the AVD_O used was the mean of the values measured just before and after 2 3 min of desaturation

In the second main series eries B) CBF was calculated from AVD_O assuming constant CMR_O Blood hemoglobin concentrat was reduced to 1 9 6 and 3 g $(100\ ml)^{-1}$ respectively A control group was obtained by withdrawal of 5 10 ml of blood and substitution with the same amount of donor blood In these animals arterial and ce ebral venous total oxygen contents (T_O) were measured - 5 15 and 30 min following the end of the bleeding substitution procedure In order to allow cal ulation of CBF changes

TABLE I Effect of acute normovolemic anemia on physiological parameters.

Number of animals	Hb content g (100 ml)$^{-1}$	Body temp C	Arterial pressure	Pao mm Hg	Paco mm Hg	pH
6	14 8±0 4	36 8±0.2	143±.	140±6	37 3±0 1	7 38 0
6	9 0±0 3	36 9±0 1	139±4	139±5	38 5±0 6	7 403±0 0
5	5 7±0 3	36 9±0 .	134±3	1 9±5	38 7±0 4	7 401±0 01
6	3.3±0 6	36 7±0 1	119±4	14.±5	35 0±0 5	7 373±0 00

In each individual animal, AVDo was also measured prior to reduction in hemoglobin content CBF in anemia (a) was then derived (in per cent of control) from AVDo before anemia (c) as

$[CBF]_a = 100\ [AVDo_2]_c\ [AVDo_2]_a^{-1}$

At each sampl ng occasion venous blood was also collected for measurements of Po and arterial blood for measurements of hemoglobin content Po Pco and pH

In both series repeated withdrawal of arterial and venous blood during the measurements of CBF and CMRo necessitated replacement via the venous catheter In order to maintain the hemoglobin content constant the hematocrit of the infused blood was properly adjusted by mixing plasma and whole blood.

Arterial and venous Po Pco and pH were measured with microelectrodes operated at 37 C with due corrections for any differences in temperature between animals and electrodes. Blood hemoglobin content was measured photometrically with a Vitatron Hb-meter Arterial and enous To was measured polarographically on 75 μl samples (Fabel and Lübbers 1964 see also Borgström *et al* 1974 b)

Statistical differences were calculated with the student s t test The following symbols are used p<0 05= p<0 01= and p<0 001=

Results

Series A

In this series the hemoglobin concentration was reduced to values of about 9 6 and 3 g (100 ml)$^{-1}$ respectively for 30 min before CBF was measured with ^{133}Xenon and CMR$_O$ was calculated Table I gives the physiological parameters Body temperature was close to 37 C in all groups At the lowest hemoglobin concentrations the blood pressure tended to

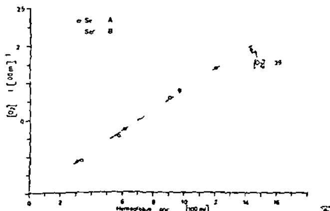


Fig. 1 Relationship between hemoglob n content and amo nt of oxygen bound to hemoglob n Each point represents the mean of 4-5 rats at -4 different times. The st ppled line is that theoreti cally calculated for an oxygen carry ng capacity for hemoglob n of 1 39 ml O$_2$ (g Hb)$^{-1}$

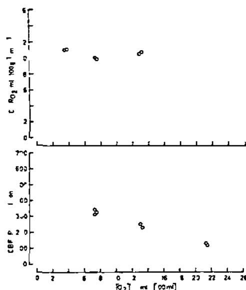


Fig. 2 Individual values for cerebral blood flow (CBF) and cerebral metabolic rate of oxygen (CMR_{O_2}) for different levels of arterial oxygen content ($[O_2]_a$) in anemia

fall somewhat but all animals had a blood pressure of 110 mm Hg, or higher. At the lowest hemoglobin concentration there was a tendency towards a fall in Pa_{CO_2} and a moderate nonrespiratory plasma acidosis. In general, however, the animals can be considered to be close to normal with respect to body temperature, blood pressure, P_{CO_2} and pH, and all had a Pa_{O_2} exceeding 110 mm Hg.

Fig. 1 gives the relationship between hemoglobin concentration and the amount of oxygen chemically bound to hemoglobin. The T_{O_2} was obtained by subtracting from the measured T_{O_2} values the amount of physically dissolved O_2, using the P_{O_2} measured and the solubility coefficient reported by van Slyke *et al* (1928). The stippled line gives the relationship which would result if each g of hemoglobin binds 1.39 ml of O_2 at full saturation. The agreement between this line and the experimentally determined T_{O_2} shows that the oxygen carrying capacity of the blood was reduced in proportion to the reduction in hemoglobin concentration.

CBF was evaluated using the same criteria as was adopted previously for hypoxic hypoxia (see Johannsson and Siesjo 1974 b). Fig. 2 illustrates the individual CBF and CMR_{O_2} values, related to arterial T_{O_2}. With reduction in hemoglobin content CBF increased gradually to

TABLE II Effect of acute normovolemic anemia on arterial O_2 content, arteriovenous difference in O_2 content, CBF and CMR_{O_2}

Number of animals	$[O_2]_a$ ml $(100\ ml)^{-1}$	AVD_{O_2} ml $(100\ ml)^{-1}$	CBF ml $(100\ g\ min)^{-1}$	CMR_{O_2} ml $(100\ g\ min)^{-1}$
6	[illegible]18 ± 0.7[illegible]	9.[illegible]5 ± 0.71	114 ± 6	10.3 ± 0.3
6	13.11 ± 0.3[illegible]	5.5[illegible] ± 0.33	[illegible]05 ± 16	11.2 ± 0.9
5	8.07 ± 0.36	3.[illegible] ± 0.3	334 ± 18	10.6 ± 0.7
6	4.83 ± 0.60	2.51 ± 0.50	510 ± 40	10.9 ± 0.6

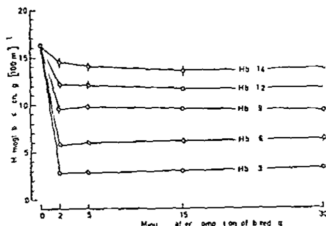


Fig 3 Hemoglobin concentration as a function of time after completion of bleeding As indicated in the figure the anemic levels chosen were 14 1., 9 6 and 3 g Hb (100 ml)$^{-1}$ Each point represents the mean±S E.M of 4-5 values.

reach maximum values of 500-600 ml (100 g) $^{-1}$ min^{-1} In anemic rats CMR$_O$ showed a larger scatter than in the control group but the data indicate that CMR$_{O_2}$ remained constant. In order to allow statistical comparisons arterial T_O AVD$_O$ CBF and CMR$_{O_2}$ were calculated for the groups given in Table I (see above) Table II illustrates the decrease in arterial T_{O_2} and in AVD$_O$ and the increase in CBF with decreasing hemoglobin content, and shows that CMR$_{O_2}$ remained constant

Series B

Since the results of series A showed that CMR$_{O_2}$ remained constant even if the hemoglobin concentration was reduced to 3 g (100 ml)$^{-1}$ changes in CBF in anemia were calculated from AVD$_O$ at 2 5 15 and 30 min following a decrease in hemoglobin content to about 12, 9 6 and 3 g (100 ml)$^{-1}$ respectively Physiological variables (body temperature blood pressure arterial P_O P_{CO} and pH) were similar to those of series A Fig 3 illustrates the hemoglobin concentration in the groups as a function of time In the control group in which 5-10 ml blood was withdrawn and an equal amount of fresh donor blood given the hemoglobin concentration decreased from a mean value of 16 g (100 ml)$^{-}$ to levels of about 14 g (100 ml)$^{-1}$ In the other groups the hemoglobin contents were after substitution close to 12 9 6 and 3 g (100 ml)$^{-}$ respectively

The percentage changes in CBF as calculated from the AVD$_O$ are illustrated in Fig 4 In the control group the CBF did not change in spite of the reduction in hemoglobin content possibly because the reduced viscosity nullified any tendency towards a small reduction in CBF with time (see Borgstrom *et al* 1974 a) In the other groups CBF increased in proportion to the reduction in hemoglobin content with maximal increases in flow at any one hemoglobin content being observed already at 2 min Since CBF did not vary with time the values for 2, 5 15 and 30 min were pooled Calculations then showed that there was a highly significant increase ($p < 0.01$) in CBF already at a reduction in hemoglobin content to 12 g (100 ml)$^{-1}$ Fig 5 shows the relationship between hemoglobin content and CBF for pooled values and compares CBF values obtained from changes in AVD$_{O_2}$ (series B filled ci to those directly measured (series A open circles) There was a fair agreement at a

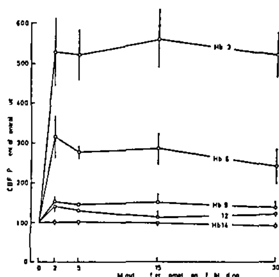


Fig 4 Changes in cerebral blood flow as calculated from the arteriovenous differences in oxygen content at different duration of anemia The gross Hb levels are indicated in the figure The values are means ± S E for groups of 4–5 animals

globin content of 9 and an excellent agreement at hemoglobin contents of 6 and 3 g (100 ml)$^{-1}$

In order to obtain indirect estimates of tissue oxygenation the cerebral venous P_O was measured in all animals of series B In addition the venous oxygen saturation was estimated in all animals of series A and B from the equation 100 $[T_O]$ $[T_O]$$^{-1}$ where a and v refer to arterial and venous blood respectively Fig 6 shows arterial T_O and venous P_O as functions of time There were minor reductions in venous P_O in the control group and in those with hemoglobin contents of 12 9 and 6 g (100 ml)$^{-1}$ However since the reduction in Pv_O was as large in the control group as in the anemic groups since mean Pv_O never

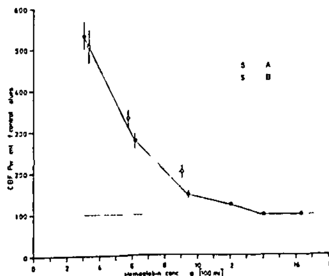


Fig 5 Relationship between hemoglobin concentration and pooled cerebral blood flow (CBF) values at different levels of anemia Values are means ± S E for 45 rats at 4 different times For further explanation see text

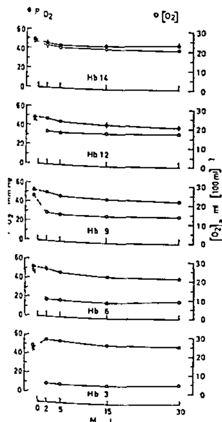


Fig 6

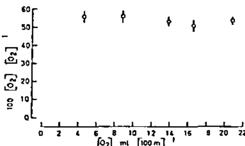


Fig 7

Fig. 6 Arterial oxygen content ($[O_2]$) and venous oxygen tension (P_vO) as functions of the duration of anemia after completion of bleeding Each point represents the mean ± S E. for 3–5 values

Fig. 7 Venous oxygen saturation (100 $[O_2]$ $[O_2]_a^{-1}$) calculated under the assumption that the normal arterial blood is 100 saturated as a function of the arterial oxygen content $[O_2]$ Each point represents the means ± S E. of 4–5 rats at 4 times (5 15 and 30 min)

fell below 40 mm Hg, and since no reduction at all was observed with a hemoglobin content of 3 g (100 ml)$^{-}$ it is concluded that the induction of anemia did not cause a reduction in venous oxygen tension Fig 7 shows that also the venous oxygen saturation remained constant and the data thus give no indication that oxygen supply to the tissue was impaired (see Discussion)

Discussion

Several previous studies have shown that normovolemic anemia, induced by withdrawal of blood and replacement with either homologous plasma or other substitution fluids induces an increase in cerebral blood flow at an unchanged CMR$_O$ (Haggendal and Norback 1966 a and b Michenfelder and Theye 1969 Paulson *et al* 1973) The present results conf these studies and demonstrate that CMR$_O$, remains unchanged even if the

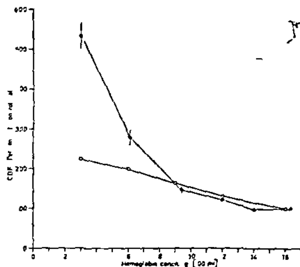


Fig 8 Relationship between changes in cerebral blood flow (CBF) and hemoglobin concentration for pooled values at different levels of anemia (filled circles) Lower line (unfilled circles) gives the theoretical CBF values which would be obtained if CBF varied with viscosity alone (cf Fig 5)

content and the arterial T_O are reduced to about 20 per cent of normal In this respect acute anemic hypoxia is thus similar to hypoxic hypoxia (see Jóhannsson and Siesjo 1974 a and b) In both conditions a normal CMR_O is maintained by means of an increase in cerebral blood flow which compensates for the reduction in arterial oxygen content However there are differences between the two conditions and the present results indicate that cerebral oxygenation is better upheld in anemic than in hypoxic hypoxia Thus although the maximal increase in CBF amounts to about 500 per cent of normal at an arterial T_{O_2} of 4–5 ml (100 ml)$^{-1}$ whether or not this reduction is due to anemic or hypoxic hypoxia, neither the venous P_O nor the venous oxygen saturation is decreased in anemic hypoxia In this respect anemic hypoxia thus seems to be characterized by perfect oxygen homeostasis i.e by an increase in CBF that does not only maintain a normal CMR_O but also normal conditions for oxygen diffusion from blood to tissue

Two facts favour adequate oxygenation of the brain in anemic hypoxia First the arterial P_O is normal and there is thus a normal P_{O_2} gradient between blood and tissue Second anemia reduces the viscosity of the blood thereby favouring an increase in CBF even if the vascular diameter does not change There is some disagreement on the effect of viscosity One group of workers (Haggendal and Norback 1966 a and b) concluded that since CBF did not vary when the hematocrit was changed between 70 and 30 per cent the increase in CBF below a hematocrit of about 30 per cent should be caused by cerebral hypoxia However Paulson *et al* (1973) recorded an increase in CBF at an unchanged venous P_O and therefore concluded that viscosity significantly influences CBF even when no tissue hypoxia is present

In the present experiments there was a small but highly significant increase in CBF at a hemoglobin content of about 12 g (100 ml) and at 9 g (100 ml)$^{-1}$ CBF was increased to at least 140 per cent of normal These results strongly indicate that viscosity changes must play a significant role in the hyperemic response Thus although increases in CBF also occur when the arterial P_O is mod rately reduced (Borgström *et al* 1974 a) such changes may possibly be explained by the effect of changes in P_{O_2} on the activity of carotid

body chemoreceptors (Ponte and Purves 1974) The influence of viscosity on CBF at con a1 vascular diameter cannot be accurately calculated since viscosity changes with vessel d a meter and rate of blood flow However approximate predictions can be made fro Poiseuille s law if one uses values for absolute viscosity at various hematocrit values on the assumption that the range of flow rates observed in control and anemic rats do not by themselves influence viscosity Fig 8 compares the percentage changes in CBF which would be obtained if flow varied with viscosity alone (unfilled circles) and the CBF values that were measured in the anemic rats (filled circles) Apparently a reduction in viscosity can explain the increase in CBF observed at hemoglobin contents of 12 and 9 g (100 ml) respectively but at lower hemoglobin contents the increase in flow is much too large to be accounted for by changes in viscosity alone

The marked increase in CBF occurring at hemoglobin concentrations of less than 9 g (100 ml) ¹ is obviously caused by other factors than reduced viscosity and must involve dilatation of cerebral resistance vessels The cause of this dilatation is not readily apparent There are two current theories explaining the cerebral hyperemia in hypoxic hypoxia According to one of these the vasodilatation is caused by tissue hypoxia which induces lactic acidosis and an increase in extracellular H^+ activity (Betz and Heuser 1967 Lassen 1968 Kogure *et al* 1970) The other theory assumes that the vasodilatation is reflexly elicited by impulses from chemoreceptors in the carotid body (Ponte and Purves 1974 for a discussion see also Borgstrom *et al* 1974 a) Neither of these theories fit the present results. Thus since venous P_O and oxygen saturation remained normal in the present anemic rats it would seem that tissue hypoxia cannot have been present Secondly the carotid chemoreceptors are believed to respond to changes in arterial P_{O_2} and there are no indications that a reduction in arterial T_{O_2} at constant P_{O_2} can trigger increased chemoreceptor activity Further experiments are needed to clarify this problem It seems especially warranted to study whether or not an unchanged cerebral venous P_O or oxygen saturation is synonymous with a normal oxygenation of brain cells under the special conditions of a pronounced anemic hypoxia

Th s study was supported by grants from the Swedish Medical Research Council (projects No 14X 63 and 14X 2179) from the Swedi h Bank Tercentenary Fund and f om U S PHS Grant No 5 RO1 NSO 7838 06 from NIH

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Brain Energy Metabolism in Anesthetized Rats in Acute Anemia

By

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Received 28 October 1974

Abstract

JÓHANNSSON H and B K SIESJÖ *Brain energy metabolism in anesthetized rats in acute anemia* Acta physiol scand 1975 *93* 515–524

In order to evaluate if pronounced anemic hypoxia gives rise to signs of cerebral oxygen lack the blood hemoglobin content was reduced to 6 and 3 g (100 ml)$^{-1}$ Cerebral blood flow increased in spite of the fact that there was a moderate reduction in blood pressure and in mean tissue CO_2 tension and in the absence of signs of an increased glycolytic rate in the tissue With a reduction in a hemoglobin content to 3 g (100 ml)$^{-1}$ there was a moderate increase in tissue lactate content and associated changes in other carbohydrate metabolites and in amino acids of the type seen in hypoxic hypoxia suggesting that tissue hypoxia was present However since the concentrations of phosphocreatine and adenine nucleotides remained constant this hypoxia must have been slight. It is concluded that there is cerebral vasodilatation in the brain in pronounced anemic hypoxia and that this vasodilatation in combination with the reduced viscosity creates favourable conditions for cerebral oxygenation

In a preceding communication (Borgstrom *et al* 1974 b) results were reported which showed that the cerebral blood flow (CBF) of lightly anaesthetized rats was progressively increased when the blood hemoglobin content was reduced from a normal value of 14–16 g (100 ml)$^{-1}$ to 12 9 6 and 3 g (100 ml)$^{-1}$ At hemoglobin contents of 12 and 9 g (100 ml)$^{-1}$ the increase in CBF could at least theoretically be attributed to reduction in blood viscosity but with more pronounced degrees of anemia the increase in CBF was far in excess of that which could have been due to viscosity changes This suggests that the cerebral hyperemia occurring at marked degrees of normovolemic anemia is related to the fall in arterial oxygen content (C_{O_2}) *i e* to hypoxia However since neither the cerebral venous P_{O_2} nor the venous oxygen saturation decreased significantly (Borgstrom *et al* 1974 b) the data failed to give evidence of the presence of cerebral hypoxia

In the present communication we report on experiments in which the blood hem content was decreased to 6 and 3 g (100 ml)$^{-1}$ respectively with subsequent

of organic phosphates glycolytic and citric acid cycle intermediates and of amino acids in the cerebral cortex. The objective of the study was to find out whether or not signs of tissue hypoxia develop at pronounced degrees of anemia *A priori* the presence of a normal venous P_O and a normal venous oxygen saturation would seem to exclude the possibility of cerebral hypoxia However experiments on arterial hypotension combined with moderate hypercapnia show that signs of energy failure in the brain may appear at normal or super normal venous P_O values (Eklof *et al* 1973) Such paradoxical results suggest that under certain conditions cerebral blood flow may become grossly inhomogeneous and that an elevated venous P_O may reflect hyperemia in only part of the tissue (Siesjo *et al* 1973) It is evident that provided such conditions arise only direct analyses of labile cerebral metabolites can reveal the presence of tissue hypoxia

Methods

As in the previous study (Borgström *et al* 1974 b) the experiments were performed on male Wistar rats (310–410 g) that were initially anesthetized with 2–3 [illegible] halothane and then maintained artificially ventilated on 70 [illegible] N_2O and 30 [illegible] O_2, with halothane administration discontinued The body temperature was adjusted to 37 C and the arterial P_{CO_2} to 35–40 mm Hg. Both femoral arteries and one femoral vein were cannulated allowing measurements of arterial P_{O_2}, P_{CO_2} pH oxygen content (T_O) hemoglobin content, glucose lactate and pyruvate as well as bleeding from the artery and isovolemic substitution with fresh homologous plasma intravenously

There were two experimental series. In one (series A) the posterior part of the superior sagittal sinus was exposed through a small burr hole allowing sampling of cerebral venous blood In these animals the blood hemoglobin content was reduced to about 6 g $(100\ ml)^{-1}$ Two and thirty min after that the procedure of bleeding and substitution had been completed arterial and cerebral venous blood were sampled where after the tissue was frozen *in situ* with liquid nitrogen (see Pontén *et al* 1973) Blood was analysed for P_{O_2}, P_{CO_2} and pH and cortical tissue for organic phosphates and for some carbohydrate metabolites The main objective of the analyses on blood was to find out whether or not the increase in CBF (as evaluated from the arteriovenous difference in T_O) was accompanied by signs of tissue acidosis due to CO_2 retention or to accumulation of organic acids. In this series no control group was studied and the metabolite data were compared to a control series of animals the brains of which were extracted at the same period using identical techniques

Since the results of series A showed that the increase in CBF could not be explained by accumulation of carbon dioxide or of organic acids a second series (series B) was made with reduction of the hemoglobin content to about 3 g $(100\ ml)^{-1}$ In these animals, the tissue was frozen *in situ* either 2 or 30 min following the end of the bleeding-substitution procedure but no cerebral venous blood was sampled However in these groups cisternal CSF was sampled for measurements of glucose lactate and pyruvate A control group was obtained by withdrawing about 10 ml of blood and substituting an equal volume of fresh blood from donor rats and by freezing the tissue about 15 min later Since the purpose of these experiments was to estimate the degree of tissue hypoxia if any measurements were made of organic phosphates glycolytic and citric acid cycle metabolites and some amino acids

Arterial and venous P_{O_2}, P_{CO_2}, pH T_O and hemoglobin content were measured as described previously (Borgström *et al* 1974 a and b) Glucose lactate and pyruvate in arterial blood and in CSF were analysed enzymatically (see below) Cortical tissue was extracted at [illegible] C and enzymatic fluorometric techniques (Lowry and Passonneau 197[illegible]) were used to measure glycogen, glucose glucose-6-phosphate (G-6-P) fructose 1 6-diphosphate (FDP) dihydroxyacetone phosphate (DHAP) 3-phosphoglycerate (3-PG), pyruvate lactate citrate α-ketoglutarate (α-KG) malate aspartate glutamate glutamine GABA, ammonia (NH_4^+) phosphocreatine (PCr) creatine (Cr) ATP ADP and AMP For references to individual methods the reader is referred to a recent communication (Folbergrová *et al* 1974)

Statistical differences were evaluated with Student's t test. The following symbols are used $p < 0.05$ = [illegible], $p < 0.01$ = [illegible] and $p < 0.001$ = *

TABLE I Hemoglobin (Hb) content mean arterial blood pressure (MABP) and art rial P_{CO} animals before and after (2 and 30 min) induction of anemia (series A)

Experimental group	Hb content g $(100\ ml)^{-1}$	MABP mm Hg	Pa_{CO} mm Hg
Control period	14 5±0 3	163±5	37.5±0 8
2 min of anemia	5 8±0 6	147±3	37 7±1 4
Control period	15 6±0 6	156±5	37 4±0 8
30 min of anemia	5 8±0 3	138±7	37 8±1.2

Results

A. *Reduction in hemoglobin content to 6 g (100 ml)⁻*

In these groups withdrawal of blood and substitution with equal volumes of hemologous plasma was performed so as to reduce blood hemoglobin content to about 6 g (100 ml)⁻ and groups of animals were studied either 2 or 30 min following the end of the dilution procedure

Table I shows the hemoglobin content mean arterial blood pressure and arterial P_{CO} before and after the induction of anemia In both anemic groups the Hb content was reduced from about 15 to a mean value of 5 8 g $(100\ ml)^{-1}$ The mean arterial blood pressure fell by about 20 mm Hg and the Pa_{CO} remained constant Body temperature was close to 37 C in all animals and arterial P_O was well above 100 mm Hg Table II shows the corresponding changes in arterial and venous T_O in CBF the latter calculated on the assumption of an unchanged cerebral metabolic rate for O_2 (CMR_O see Jóhannsson and Siesjo 1974 Borgstrom *et al* 1974 b) and in Pv_O CBF increased to more than 300% at 2 min and to 234 at 30 min The lower value at 30 min was probably due to the fact that 3 values for AVD_O measured before induction of anemia, were lower than 8 ml $(100\ ml)^{-1}$ The results confirm our previous report (Borgstrom *et al* 1974 b) in showing that at this degree of anemia, CBF increased 2 to 3 fold in spite of an unchanged cerebral venous P_O Furthermore they demonstrate that the increase in CBF occurred at an unchanged Pa_{CO}, and in spite of a fall in mean arterial blood pressure Thus neither a rise in Pa_{CO} nor in perfusion pressure could have caused the cerebral hyperemia

The arteriovenous difference in P_{CO} (ΔP_{CO}) between femoral artery and superior sagittal sinus was measured before and after the induction of anemia in 7 animals (3 animals at 2 min and 4 at 30 min) Before anemia ΔP_{CO} was 10 1 mm Hg ± 0 9 (mean ± S E) a value which is in good agreement with previously reported ones (see Brzezinski *et al* 1967 MacMillan and Siesjo 1972) In the anemic animals the corresponding value was 6 8 ± 0 7 mm Hg This value is significantly different from the controls ($p < 0\ 05$) Since the Pa_{CO} remained constant there must have occurred a fall in tissue CO_2 tension If the mean tissue CO tension (Pt_{CO}) is derived from the equation (Ponten and Siesjo 1966)

Table II. Total oxygen content (O) in arterial and venous blood, venous oxygen tension (PvO) and cerebral blood flow in % (CBF) before and after 2 and 30 min of anemia. Hb-content 5 8 g (100 ml)$^{-1}$ (Series A.) n = 6 in both groups.

Experimental group	$[O_2]_a$	$[O_2]$ ml (100 ml)$^{-1}$	PvO_2 mm Hg	CBF %
Control period	20 13 ± 0.5	9 60 ± 0 91	49 1 ± 2.6	100
2 min of anemia	7.85 ± 0 49	4 60 ± 0 69	48.9 ± 1 7	3_8 ± _1
Control period	21 66 ± 1.21	11 74 ± 0 63	44.8 ± 2.9	100
30 min of anemia	8.60 ± 0.35	4 14 ± 0.2	41 7 ± 2.6	234 ± _6

it can be calculated that Pt_{CO_2} fell by about 1 mm Hg. Therefore the increase in CBF cannot have been caused by CO_2 retention in the tissue (see Discussion)

As stated in the introduction, tissue metabolites in the anemic animals were compared to a control group which was not run or extracted simultaneously Small differences in concentrations for individual metabolites should therefore be interpreted with some caution and the results mainly serve as rough guidelines. Table III gives the concentrations of PCr creatine, ATP ADP AMP Lactate and pyruvate Clearly there were no changes in organic phosphates suggestive of energy failure in the anemic animals and there were only moderate changes in lactate and in lactate pyruvate ratio A comparison between the control and anemic animals in tissue concentrations of glycogen, glucose, G-6-P pyruvate citrate α KG and malate showed that the anemic animals had a higher glucose content but all other metabolites were similar A corresponding agreement was obtained for glutamate glutamine aspartate and NH (not shown)

The increase in the tissue contents of lactate and glucose could not be directly ascribed to an effect of the anemia on tissue metabolism. Thus, the blood contents of glucose and lactate were markedly increased in the anemic animals. It was thus a possibility that the corresponding tissue changes merely reflected in a passive manner the events occurring in blood. However since CSF was not analysed the comparison between changes in tissue and extracellular fluids was postponed until animals with a Hb content of 3 g (100 ml)$^{-1}$ had been analysed. Tentatively we interpret the metabolic pattern observed in the animals with a Hb content of 6 g (100 ml)$^{-1}$ to indicate that tissue metabolism was not altered as a result of the anemia.

B Reduction in hemoglobin content to 3 g (100 ml)$^{-1}$

In this series there were 4 animals in each group (control, 2 and 30 min of anemia at constant Hb content). Table IV shows that the Hb content was reduced to about 3 g (100 ml)$^{-1}$ in the anemic groups, as compared to 16–17 g (100 ml)$^{-1}$ in the control periods The Pa_{CO_2} fell somewhat with time in the 2 min group but the values were similar in the anemic groups at the time of sampling of tissue and CSF In the separate control group Pa_{CO_2} was 36 9 ± 0 6 mm Hg at the time of sampling tissue and CSF Body temperature remained close to 37 C in all animals, Pa_{O_2} was in excess of 100 mm Hg, and plasma pH remained normal (not shown).

Table V demonstrates the lactate and pyruvate concentrations in tissue CSF and blood.

TABLE III Cerebral tissue concentrations of phosphocreatine (PCr) creatine (Cr) ATP ADP [illegible] lactate and pyruvate (μmol g⁻¹) 2 and 30 min after the reduction in Hb content to 6 g (ml)⁻¹ The values are compared to those obtained in a control group of a separate study [illegible] ±S E 6 animals in each group

Experimental group	PCr	Cr	ATP	ADP	AMP	Lactate	Pyru ate
Control period	4 58±0 08	5 94±0 10	3 06±0 0_	0 275±0 011	0 038±0 001	1 48±0 04	0 11 ±0 004
2 min of anemia	4 92±0 12	6 01±0 08	3 09±0 005	0 273±0 008	0 0?8±0 001	1 90±0 14	0 1 6±0 007
30 min of anemia	4 81±0 11	6 09±0 01	3 09±0 01	0 283±0 017	0 0 7±0 003	1 99±0 17	0 117±0 004

The tissue concentration of lactate and the lactate/pyruvate ratio were moderately increased in the anemic groups but the pyruvate concentration was unchanged Relatively more pronounced increases in lactate and lactate/pyruvate ratio were observed in arterial blood and also in CSF In blood there was a significant increase in pyruvate as well In order to allow calculation of intracellular lactate and pyruvate concentrations it was assumed that the blood volume of the tissue was 3 of the tissue weight and the extracellular volume 15° This correction for extracellular contents of the metabolites indicates that there were in fact increases in intracellular lactate concentration and lactate/pyruvate ratio However the increases were moderate and statistically significant ($p < 0\ 05$) only at 2 min

Table VI gives the corresponding values for glucose In anemia glucose in the tissue increased significantly However glucose in the blood which was high already in the control group rose to excessively high values in the anemic animals Thus since the tissue to blood glucose concentration ratio was the same in all three groups it is indicated that the increase in the intracellular glucose concentration in the anemic animals was secondary to hyper glycemia

Fig 1 illustrates the percentage changes in glycolytic and citric acid cycle intermediates in the anemic animals All control values are in μmol (g of fresh weight)⁻ except for glucose and pyruvate which were derived for intracellular water assuming a 15 ₒ extracellular fluid volume At 2 min there were moderate increases in glucose G-6-P F 6-P citrate ᵅKG and malate and a fall in 3 PG concentration This pattern of changes is similar to that observed in hypoxic hypoxia except for the fact that pyruvate did not increase in the anemic animals (see Discussion) At 30 min a similar pattern was observed but statistically significant changes were present only for glucose G 6-P F 6-P and 3 PG At no time were there changes in glycogen FDP DHAP pyruvate succinate or fumarate

TABLE IV Haemoglobin (Hb) content mean arterial blood pressure (MABP) and arterial P_{CO} in anemic animals before and after (2 and 30 mi) reduction of the Hb co tent (series B)

Experimental group	Hb content g (100 ml)⁻	MABP mm Hg	Pa_{CO} mm Hg
Control period	16 8±0 3	153±6	39 1+0 4
2 min of anemia	3 0±0 3	1 5±3	36 3±0 6
Control period	16 6±0 5	148±5	36 9±1 0
30 min of anemia	3 ±0	1 5±3	36 3±0 7

TABLE V Levels of lactate and pyruvate in blood CSF and cerebral cortex measured in control and anemic (2 and 30 min) groups Hemoglobin content was 3 g (100 ml)[1] in the anemic groups (series B) Lactate and pyruvate concentrations are given in μmol g⁻¹ (of wet tissue or intracellular water) Means ± S E. 4 animals in each group The intracellular concentrations were calculated from the concentrations in tissue CSF and blood using a tissue water content of 0 79 g per g of tissue and assuming extracellular and blood volumes of 15 and 3 respectively p < 0 05 – p < 0 01 – and p < 0 001 –

Experimental g oup	Tissue		CSF		Blood		Intracellular water		
	La	Py	La	Py	La	Py	La	Py	La/Py
Control group	1 67 ±0 03	0 1⌞0 ±0 004	2 14 ±0 03	0 174 ±0 003	2 38 ±0 12	0 170 ±0 008	2 09 ±0 06	0 145 ±0 006	14 8 ±0 8
2 min of anemia	2.29 ±0 12	0 131 ±0 004	2 94 ±0 10	0 177 ±0 006	7 18 ±0 95	0 291 ±0 018	2 68 ±0 15	0 157 ±0 007	17 ˀ ±0 8
30 min of anemia	2 44 ±0 22	0 130 ±0 006	3 71 ±0.2	0 174 ±0 003	7 51 ±0 80	0 265 ±0 017	2 72 ±0 ˏ9	0 158 ±0 009	17 1 ±1 1

Changes in amino acids are illustrated in Table VII There was no change in glutamate but the data suggest that aspartate and GABA fell in the anemic animals there was a significant rise in glutamine at 30 min and there were clear increases in alanine These changes are almost identical to those observed in hypoxic hypoxia (see Discussion) NH_4 remained constant in anemia (the values were 0 22 ± 0 03 0 24 ± 0 02 and 0 26 ± 0 03 in the control and anemic groups respectively)

The tissue concentrations of organic phosphates are given in Table VIII There were changes in PCr creatine ATP ADP or AMP in the anemic animals and it should be specially noted that the PCr concentration remained at 5 μmol g⁻¹

The constancy in the PCr/Cr and ATP/ADP ratios suggest that intracellular pH (pH_i) remained constant In order to estimate pH_i the apparent equilibrium constant for the creatine phosphokinase (CPK) reaction was first derived using the mean ATP/ADP and PCr/Cr ratios for the control group and an assumed normal pH_i of 7 044 (Siesjo *et al* 1972) Using this equilibrium constant pH_i was then calculated from the ATP/ADP and PCr/Cr ratios in each individual rat The values derived for the control and the anemic

TABLE VI Levels of glucose in blood CSF and cerebral cortex measured in control and anemic groups Glucose values are gi en in μmol g⁻¹ (of wet weight or intracellular water) Means S E. 4 animals in each group The intracellular glucose concentrations were cal ulated from the glucose concentrations in tissue CSF and blood using a tissue water content of 0 77 g per g of tissue and assuming extracellular and blood volumes of 15 and 3 respecti ly

Experimental group	Tissue Glucose	CSF Glucose	Blood Glucose	Intracellular water Glucose
Control period	3 85 0 37	6 09 ‿0 65	14 30 ± 0 83	4 5 ±0 08
2 min of anemia	5 89 0 75	6 5 0 33	3 6±‿79	6 90 ‿1 0
30 min of anemia	7 84 ‿0 3	8 79 ‿0 46	6 58 ± 1 91	8 80 ‿0 61

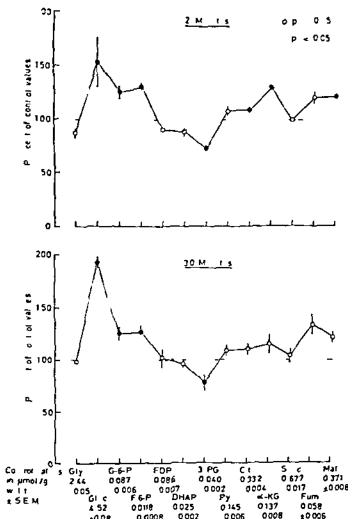


Fig. 1 Influence of anemia (he noglobin conte t 3 g (100 ml)$^{-}$) on glycolytic and citric ac d cycl metabolites in c rebral cort cal tissue The values are gi en as per cent of controls (± S E.) w th absol te v lues for cont ol group shown at the bottom Filled symbols denote alues sign ficantly different from controls ($p < 0\,05$)

groups were 7 036 ± 0 005 7 023 ± 0 016 and 7 023 ± 0 018 respectively In other words calculations from the CPK equilibrium indicate that pH_i remained constant within 0 02 units in anemia

Discussion

As stated in the introduction cerebral venous P_O is an ambigous parameter for the evalua tion of the presence of cerebral hypoxia and analyses of cerebral metabolites are required The metabolic changes occurring in hypoxic hypoxia have been mapped out in some detail (Bachelard *et al* 1974 Norberg and Siesjö 1975 a and b Siesjo *et al* 1974) Such

TABLE VII. Cerebral tissue concentrations of glutamate (Glut), glutamine (Glu n), aspartate (Asp), γ amino-butyric acid (GABA) and alanine (Aln) in control and anemic groups ($\mu mol\ g^{-1}$). Values are means ± S.E. for groups of 4 animals. $p < 0.05 = *$, $p < 0.01 = **$ and $p < 0.001 = ***$

Experimental group	Glut	Glu n	Asp	GABA	Ala
Control	13.07 ± 0.31	6.05 ± 0.11	3.67 ± 0.07	2.42 ± 0.06	0.454 ± 0.01[illegible]
Anemia 2 min	13.51 ± 0.16	5.61 ± 0.40	3.34 ± 0.09	2.21 ± 0.09	0.539 ± 0.0[illegible]0
Anemia 30 min	13.58 ± 0.74	6.84 ± 0.[illegible]3	3.31 ± 0.18	2.09 ± 0.09	0.580 ± 0.0[illegible]

have shown that relatively pronounced degrees of hypoxia are required to induce changes in cerebral energy state. Thus, when the Pa_{O_2} is reduced to 30 mm Hg or lower, there is a fall in PCr and a small rise in ADP, suggestive of moderate imbalance between production and utilization of ATP. However, changes in lactate content are more easily detected and such changes are accompanied by alterations in the tissue concentrations of also other carbohydrate metabolites and in some amino acids. Under semi steady state conditions, *e.g.* after 30 min of hypoxia, these changes include increases in G 6-P, F 6-P, pyruvate, lactate, citrate, α KG, fumarate, malate and alanine, and decreases in aspartate concentration. These changes are logically interpreted as being secondary to stimulation of glycolysis at the phosphofructokinase, hexokinase and possibly pyruvate kinase steps, and to increased reduction of the lactate and malate dehydrogenase systems (see Norberg and Siesjö 1975 a and b). Tentatively, the increased glycolytic rate gives rise to an elevated concentration of pyruvate and this in turn may be responsible both for the increase in alanine (via a shift in the alanine aminotransferase reaction) and for an increase in the size of the citric acid cycle pool (via an increased rate of pyruvate carboxylation). A decrease in aspartate could then be explained by a shift in the aspartate aminotransferase reaction, secondary to an effect of an altered redox state on the oxaloacetate concentration.

In the present series, only 4 animals were included in each group and the inevitable variability in results makes it difficult to detect very small changes in the concentrations of some metabolites. In the following discussion we will therefore disregard the fact that some changes were not statistically significant provided that the suggested change was consistent in the 2 and 30 min groups. Clearly, any hypoxic changes in the tissue as a result of the anemia were slight. Thus, there were no changes in any of the organic phosphates and the intracellular lactate concentrations increased by only about 0.5 $\mu mol\ g^{-1}$. However, the pattern of changes observed in other metabolites is consistent with the idea that there was a moderate change in metabolism in the direction expected to occur in hypoxia. Thus, there were increases in G 6-P and F 6-P, an increase in the pool of the citric acid cycle intermediates due to increases in citrate, α KG and malate (probably also in fumarate), a decrease in aspartate and an increase in alanine. Furthermore, the lactate/pyruvate ratios were elevated at an unchanged intracellular pH, indicating an increased reduction of cytoplasmic redox systems, and there was a small rise in glutamine at 30 min. It is not entirely clear why these changes occurred in the absence of a rise in pyruvate concentration but the small number of animals analysed may have obscured a true rise in intracellular pyruvate concentration. Therefore, we conclude that slight tissue hypoxia was present in the brains of the anemic

TABLE VIII Cerebral tissue concentration of phosphocreatine (PCr) creatine (Cr) ATP ADP and AMP (μmol g^{-1}) 2 and 30 min after the reduction in Hb-content to 3 g (100 ml)$^{-1}$ Means ± S E 4 animals in each group

Experimental group	PCr	Cr	ATP	ADP	AMP
Control period	4 98 ±0 03	5 49 ±0 07	3 11 ±0 01	0 306 ±0 003	0 031 ±0 001
2 min of anemia	4 95 ±0 08	5 45 ±0 08	3 12 ±0 03	0.305 ±0 00[illegible]	0 033 ±0 00[illegible]
30 min of anemia	5 10 ±0 08	5 72 ±0 08	3 15 ±0 04	0 305 ±0 003	0 034 ±0 001

animals in spite of the unchanged cerebral venous P_O Evidently there must be some degree of inhomogeneity in flow at very low Hb concentrations and the tissue may well contain areas that are more hypoxic than the average values for metabolites suggest However it should be stressed that the unchanged energy state precludes the presence of a true im balance between production and utilization of energy

As judged from the increase in tissue lactate content and from the accompanying changes in other carbohydrate metabolites and in amino acids anemic hypoxia with reduction in Hb content to 6 g (100 ml)$^{-1}$ is roughly equivalent to hypoxic hypoxia with reduction in Pa_O to about 45 mm Hg (see Siesjo *et al* 1974) However in hypoxic hypoxia of this degree CBF increases to less than 200°$_o$ of normal and there is a significant reduction in cerebral venous P_O (Borgstrom *et al* 1974 a) When the blood Hb content is reduced to 3 g (100 ml)$^{-1}$ CBF increases to 500–600 $_o$ of normal (Johannsson and Siesjö 1973 Borgstrom *et al* 1974 b) In order to obtain a comparable increase in CBF in hypoxic hypoxia Pa_O must be reduced to 20–25 mm Hg However at this degree of hypoxia there is a marked lactic acidosis in the tissue and a significant change in energy state (Norberg and Siesjo 1975 a) These comparisons suggest that the increase in CBF is not directly related to the degree of tissue hypoxia The results clearly indicate that the reduction in blood viscosity in anemia significantly influences the circulatory response to hypoxia. However as was pointed out in the preceding communication (Borgstrom *et al* 1974 b) the reduced viscosity can explain only part of the increase in CBF observed at Hb contents of 6 and 3 g (100 ml) and it remains to be discussed how dilatation of cerebral resistance vessels can occur in anemic hypoxia

Two hypothesis have been advanced to explain the increase in CBF that occurs in hypoxic hypoxia According to one dilatation of cerebral resistance vessels is induced by extra cellular acidosis secondary to increased cerebral glycolysis (see Lassen 1968 Kogure *et al* 1970) According to the other hypothesis cerebral vasodilatation is elicited reflexly from the carotid bodies and the hypoxic hyperemia is completely abolished if the sinus nerves are sectioned (Ponte and Purves 1974) Recent results from this laboratory are consistent with the second hypothesis Thus since the increase in CBF in hypoxic hypoxia occurs *before* the tissue has been acidified by accumulated lactic acid (Borgstrom *et al* 1974 a) a reflex mechanism would seem likely However it seems far from the clear that a similar mechanism would operate in anemic hypoxia In anemia the time cause of changes in CBF

cannot be studied since it is not practically feasible to reduce abruptly the hemoglobin content Furthermore since chemoreceptors in the carotid body are believed not to be sensitive to a reduction in blood oxygen content at constant P_O the appropriate signal is not readily apparent At the present time no plausible explanation can be given for the vascular dilatation that occurs at pronounced degrees of anemic hypoxia However whatever is the mechanism of vasodilatation the combination of vasodilatation and reduction in viscosity appear to provide relatively optimal conditions for cerebral oxygenation in spite of a pronounced fall in arterial oxygen content

This study was supported by grants from the Swedish Medical Research Council (projects No 14X 63 and 14X 2179) from the Swedish Bank Tercentenary Fund and from U S PHS Grant No 5 R01 NS07838 06 from NIH

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Acta physiol scand 1975 93 525-530
From the Institute of Physiology University of Lund Sweden

Compensatory Increase in Choline Acetyltransferase Activity in Salivary Glands and Diaphragm Muscle of the Rat

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Received 29 October 1974

Abstract

EKSTRÖM J *Compensatory increase in choline acetyltransferase activity in salivary glands and diaphragm muscle of the rat* Acta physiol scand 1975 *93* 525-530

When the function of salivary glands was abolished by applying ligatures to their ducts and the function of one half of the diaphragm muscle was abolished by sectioning of its phrenic nerve the choline acetyltransferase activity was found to be increased in not duct ligated glands and in the intact hemidiaphragm 4 weeks later The increase was not seen within the first week The increase in activity appears to be particularly manifested in the nerve endings since it was seen in the hemidiaphragm but not in the phrenic nerve Increased stream of impulses in the efferent nerves is thought to be the cause of this increase in choline acetyltransferase activity

Key word Choline acetyltransferase activity salivary glands diaphragm muscle

When the preganglionic parasympathetic salivary nerve is cut the ability to form acetylcholine in the postganglionic neurones of the gland decreases as judged from the reduction of choline acetyltransferase activity (Nordenfelt 1964 Ekstrom and Holmberg 1972 a) A fall in the activity of this enzyme of about the same magnitude can be induced in the parotid gland of the rat by keeping the animal on a liquid diet (Ekstrom 1973) The opposite *i e* a rise in the enzyme activity occurs in the gland when the rat is fed on a dry cellulose rich diet or when the animal is atropinized causing dryness of the oral mucosa (Ekstrom 1974) The results of these studies suggest that the choline acetyltransferase activity is depending on the impulse propagating activity of the neurones as originally pointed out by Nordenfelt (1964) the enzyme activity declines when the flow of secretory impulses in the neurones is lost or reduced and it increases when the impulse traffic is augmented

Observations on skeletal muscles show that disuse is followed by a decrease in choline acetyltransferase activity (Gutmann Tuček and Hanzlíková 1969 Snyder Rifenberick and Max 1973 Diamond Milfay and Franklin 1974) However opinions differ as to the effect of overuse Thus an increased choline acetyltransferase activity was found by Snyder *et al* (1973) in the plantaris muscle of the rat when the tendon of the synergistic muscles gastroc

nemius and soleus had been severed for 8 days while Diamond *et al* (1974) were unable to demonstrate any increase in the rat gastrocnemius muscle 7 days after the tendons of the soleus and plantaris muscles had been cut

When saliva is prevented from reaching the oral cavity of the rat by ligation of the ducts of some of the salivary glands the reflexly elicited stimulation of the other not duct ligated glands appears to be augmented as judged from glandular enlargement (Wells and Peronace 1964 Elmér and Ohlin 1969) and increased secretion (Elmér and Ohlin 1969) In the present study the choline acetyltransferase activity was estimated in such non ligated glands As in salivary glands (see Nordenfelt 1965 a Ekstrom and Holmberg 1972 b) the acetylcholine forming enzyme is in the diaphragm muscle confined to the cholinergic neurones (Hebb Krnjevic and Silver 1964 Emmelin Nordenfelt and Perec 1966 Israël 1970) In the present investigation the enzyme activity was determined in one half of the muscle and in its phrenic nerve when the nerve on the contralateral side of the rat had been sectioned in advance This procedure seems to lead to an increase in the stimulation of the innervated hemidiaphragm since according to the result of preliminary experiments the respiratory frequency of the rat rose

Methods

74 male rats of a Sprague Dawley strain bred at this Institute were used The rats were at the start of the experiments 3 5 months old and weighed 260–380 g They were given a standard pelleted diet and water *ad libitum* The experiments were performed as follows

Salivary glands The rats were anaesthetized with ether In the first series the parotid duct on the left side and the ducts of the submaxillary and the sublingual glands on both sides were tied with a fine silk thread the ducts of the latter two glands close to hilus causing at the same time an interruption of the parasympathetic nerve supply In the second series both parotid ducts and those of the submaxillary and the sublingual glands on the left side were ligated At the end of the experimental period the rats were killed with ether and weighed The position of the ligatures was controlled and macroscopical changes of the duct ligated glands were looked for such as dark red colour and atrophy In the first series of experiments the nonligated right parotid glands from 5 rats were examined 3 days after the ligation procedure glands from another 5 rats 4 days later and from 9 rats 78 days after the beginning of the investigation In the second series the submaxillary and the sublingual glands on the right side of 9 rats were studied after 8 days too When the glands had been removed they were cleaned washed in saline pressed gently between gauze pads and weighed Since dry weight is considered more reliable the weight of the acetone dried powder was used As control glands served the corresponding ones of litter mates to duct ligated rats in two cases the control rat was common to both the series of experiments Thus 54 rats were used

Phrenic nerve and diaphragm The rats were anaesthetized with pentobarbitone 60 mg kg i p At the same time atropine sulphate 1 mg kg i p was given in order to suppress bronchial secretion Artificial respiration was given by a pump through a fine glass tube which was inserted into the trachea through a small cut Between 2 ribs the right phrenic nerve was sectioned in the upper part of the thorax and a nerve segment of about 5 mm was removed The intrathoracic approach was chosen since some of the cervical branches forming the phrenic nerve otherwise might escape the section when done in the neck region (Sola and Martin 1953 Emmelin and Malm 1965) The wounds were sutured and the artificial respiration was stopped After 8 days the respiratory frequency of the operated rat was estimated when the animal seemed to be at rest the frequency was also determined in the litter mate used as control The rats were then killed with ether and weighed The right sectioned phrenic nerve was inspected In some instances signs of beginning nerve regeneration could be observed in the form of anatomical connection with the proximal part of the phrenic nerve or intercostal nerves The left phrenic nerve was cut close to the diaphragm and taken out. A piece of 3 mm of the distal part of the nerve was cut away The length of the nerve used was 4 cm The left hemidiaphragm was then taken out the dorsal slip being discarded The muscle was carefully cleaned washed in saline pressed between gauze pads and weighed The weight of the acetone dried powder

was used as dry weight. In this type of experiment ?0 rats were used 10 were control l tter ma es to th denervated ones.

Estimation of choline acetyltransferase activity The method de ised by Hebb (see Nordenfelt 1963 1965 a) was used Acetone dried powder was prepared from individual salivary glands, except for the sub lingual glands where 4 or 5 glands were pooled Acetone dried powder was also made from individual hemi diaphragms phrenic nerves were pooled

The powder of the glands and the hemidiaphragms was made up in cysteine-sal ne in a concentration of 50 mg ml, the powder of the phrenic nerves in a concentration of 1_ cm nerve ml. Of the tissue extracts from the glands and the nerves 0.2 ml, from the hemidiaphragms 0 4 ml, were incubated at 38 C for 60 min The incubate was assayed for acetyl hol ne on the eserinized frog rectus The chol ne acetyltransferase ac tivity is expressed in μg acetylcholine chloride formed pr h per gland or pooled glands (total acti ity) and in μg acetylcholine chloride formed per h per g acetone powder or per cm of nerve (concentration)

Student s t test was used paired comparison were made between the operated rat and its control litter mate P values of less than 0 05 were considered significant.

Results

Body weights At the start of the experiments no differences existed in body weights between the rats to be used as controls and those to be operated on When the final body weights of the rats are expressed in per cent of their initial weights the mean percentage figure obtained in the duct ligated animals 3 days after the beginning of the experiment was 3% lower ($p < 0.02$) than that in the control rats while it was the same as that of the controls 1 week and 4 weeks after the start at the latter time the body weights were found to have increased by about 7–8 The rats with denervated hemidiaphragms and their control increased in body weights by about 6 and 10% during the experimental period of 4 weeks the difference is significant ($p < 0.02$)

Salivary glands The mean choline acetyltransferase activity of the parotid glands of the whole group of control animals was when expressed per gland 23.6 ± 1.3 ($n = 19$) and per g acetone powder 703 ± 32 ($n = 19$) μg acetylcholine formed during 1 h the corresponding figures for the submaxillary glands were 17.0 ± 2.2 ($n = 9$) and 418 ± 44 ($n = 9$) As shown in Fig. 1 the total choline acetyltransferase activity of the parotid gland was 32% higher in the rat with otherwise duct ligated salivary glands than in the rat used as control, when the glands were examined 4 weeks after the ligation procedure no change in the total enzyme activity was observed during the first week After 28 days of ligation of the submaxillary and sublingual ducts on one side and the two parotid ducts the total enzyme activity in the intact submaxillary gland was al o found to be 32% higher than in the control gland The dry weights of the parotid and the submaxillary glands were 52 and 38% higher respectively than those of their controls after 4 weeks of duct ligations Already at the end of the first week the intact parotid glands were 38% heavier than the controls

In the two examined pools of sublingual glands from the not duct ligated side of the rats the total enzyme activity was 112 and 113 per cent of that of their respective pool of control glands the dry weights were 131 and 133 per cent of their control

Phrenic nerve and diaphragm The choline acetyltransferase activity per cm of left phrenic nerve and per left hemidiaphragm in the rats used as controls was 6.2 ± 0.8 ($n = 4$) and $_.5 \pm 0.5$ ($n = 10$) respectively if expressed per g acetone powder the enzyme activity of the hemi diaphragm muscle was 364 ± 5 ($n = 10$) The mean respiratory frequency of these 10 control

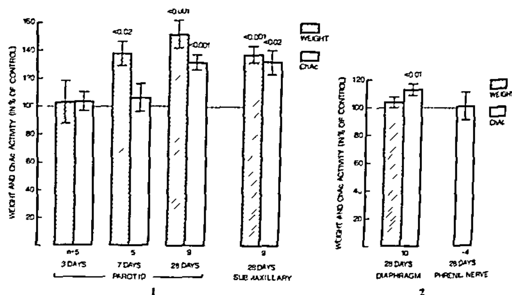


Fig. 1 Dry weight and choline acetyltransferase activity (calculated per gland) of the intact salivary glands at different time intervals after the ligation procedure The weight and the enzyme activity are expressed as a percentage of those of the gland of the control litter mate (mean ± S E.) Number of comparisons are shown and when the differences are significant the p-values are given

Fig. 2. Dry weight and choline acetyltransferase activity (calculated per hemidiaphragm) of the intact half of the diaphragm muscle and choline acetyltransferase activity of the phrenic nerve (calculated per pool of 2 nerves) after section of the nerve on the contralateral side Results are expressed as in Fig. 1

rats was 45 ± 1 breaths per 30 s The enzyme activity of the left phrenic nerve in the rats, in which the nerve on the right side had been sectioned 28 days earlier did not differ from that of the nerve in the control rats as shown in Fig. 2 However the total enzyme activity was 13% higher in the left hemidiaphragm than it was in the control muscle no change in the dry weight was observed The respiratory frequency of the operated rats was found to be higher (p 0 001) than that of the control rats when expressed in per cent of the controls it was 131 ± 3 (n 10)

Discussion

The present study shows that after 4 weeks of increased impulse traffic in efferent pathways the choline acetyltransferase activity is increased in salivary glands and in hemidiaphragm. In the earlier study in which increased reflex stimulation of salivary glands also was induced, increased enzyme activity was at hand when the glands were examined 3 weeks after the beginning of the experiments (Ekstrom 1974) It appears from the present investigation that it takes some time for the phenomenon to develop since no increase in the enzyme activity was observed in the parotid gland within the first week The secretory activity of the gland was probably increased already at the end of this week as shown by the glandular hypertrophy It is of interest to note that the increase in choline acetyltransferase activity produced in some salivary glands by sympathetic denervation also seems to be a late phenomenon, not occurring until after about 2 weeks (Nordenfelt 1965b Ekstrom 1972) A possible ex

planation to the fact that the increase in enzyme activity failed to appear in the skeletal muscle studied by Diamond *et al* (1974) might be that the time period chosen was too short to allow the increase to develop under their experimental conditions

The increase in choline acetyltransferase activity was bigger in the parotid gland than in the hemidiaphragm muscle Although it is possible to obtain long pieces of the auriculo-temporal nerve for choline acetyltransferase analysis the disadvantage with the nerve is its early branching Therefore in the present work the phrenic nerve was used No change in the enzyme activity was observed the activity in the nerve was of the same order as that of earlier investigations (Hebb *et al* 1964 Ekstrom and Emmelin 1971) It thus appears that the increase in activity of the acetylcholine synthesizing enzyme is particularly manifested in the nerve endings

Mucosal dryness and dry food were in the previous work found to affect especially the parotid gland as judged from the increase in choline acetyltransferase activity and the increase in weight (Ekstrom 1974) It is also this gland which shows the biggest glandular enlargement in the present study

No hypertrophy was found in the hemidiaphragm this is in accordance with the observation of Sola and Martin (1953)

This work was supported by a grant from the Faculty of Medicine in Lund

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Acta physiol scand 1975 93 531-539
From the Kinesiology Laboratory University of Jyväskylä Finland

Signal Characteristics of EMG with Special Reference to Reproducibility of Measurements

By

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Received 4 November 1974

Abstract

VIITASALO J H T and P V KOMI *Signal characteristics of EMG with special reference to reproducibility of measurements* Acta physiol scand 1975 *93* 531-539

Reliability and constancy of recordings of EMG signal characteristics were investigated from the measurements taken with miniature size surface electrodes during submaximal and maximal contraction of the rectus femoris muscle The following EMG variables were studied integrated EMG (IEMG) various band widths of the power spectral density function mean power frequency (MPF) and rise time amplitude and number of spikes of the averaged motor unit potential (AMUP) The results indicated that for most of the variables studied the reproducibility of measurements was better within the test session (reliability) than between the different test days (constancy) The reliability values for IEMG MPF and AMUP amplitude were rather high (r=0 77-0 9) MPF and number of spikes in AMUP showed good constancy values (r=0 73-0 93) and it is suggested that these parameters can be recommended for use in EMG studies where recordings are repeated over a period of several days

Key words Electromyography measurement reproducibility spectral analysis signal charateristics EMG amplitude

Quantitative electromyography has been used conventionally as a tool to classify the neural activity level for producing a certain muscular tension Such studies as those of Inman *et al* (1952) Bergstrom (1962) and many others have nicely shown that there exists a linear or slightly nonlinear relationship between integrated EMG activity (IEMG) and muscular tension. Some authors (Bouisset and Goubel 1968) have demonstrated how the quantified EMG activity varies with changes in several biomechanical quantities of muscle function Komi (1973) was able to show how the relationship between the neural input as measured with quantitative electromyography and muscular tension is affected highly by the velocity and type of muscle contraction

A fundamental question in EMG studies as well as in any other biological research problem is the reproducibility of measurements There are some reports in the literature (*e g* DeVries 1968 Courier 1969 Komi and Buskirk 1970 Kramer 1972) to demonstrate the reproducibility problems faced in "global" type EMG measurements Recent trends in EMG research seems to emphasize the importance of a more qualitative approach in

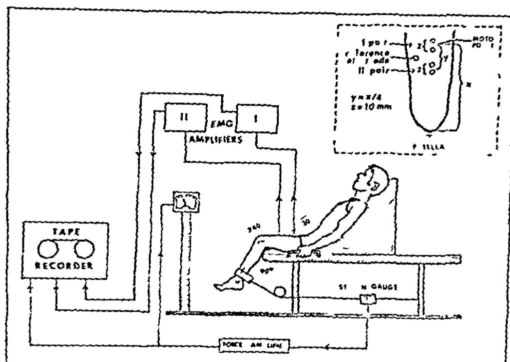


Fig. 1 Schematic diagram explaining the testing system. The insert shows the front view of the right thigh with the electrode placement on the rectus femoris muscle

nesiological electromyography Especially the use of automated computer analysis of EMG potentials have opened up new possibilities in this aspect. However in order to accept fully the new methods it is important that one tries to find how their parameters are related to physiological measures and how reliable and reproducible their measurements are

This report deals with the relationship between selected signal characteristics of EMG potential and muscular tension Particularly attention was paid to the reliability and constancy of recordings

2. Methods

Quadriceps femoris muscle was voluntarily contracted isometrically using the instrumentation system shown in Fig. 1

2.1 EMG measurements

The motor point of the rectus femoris muscle was determined with a Neuroton 6_6 stimulator ? pairs of Beckman miniature size skin electrodes of 4 mm in contact diameter were placed on the muscle so that the first pair was just above the motor point The midpoint of the second electrode pair was _5 in distance distally from the first pair along the line motor-point patella (see Fig. 1 insert) The interelectrode distance of both pairs was 10 mm. The common reference electrode was placed on the lateral side of the thigh

The skin was carefully prepared before the measurements by thorough sponging with alcohol plus rubbing with sand paper This secured that the interelectrode resistance was always below 5 K ohms. The electrode sites were marked for day to-day measurements by a drop of _0 AgNO solution as described earlier (Komi and Buskirk 1970)

The EMG signals were amplified with Tektronix RM 1__ low level preamplifier (10 dB spectrum range

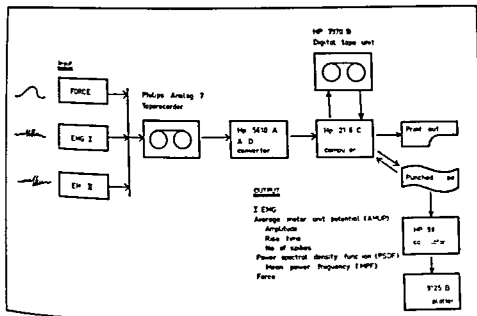


Fig. .. Data process ng system.

0 8 Hz 10 KHz) and subsequently stored in analog form on a Philips Analog 7 tape recorder The recording speed was 15 /s

2.? *Fo ce—measu ements*

The exerted force was measured w th strain gauge system installed under the dynamometer table (see Fig 1) The force signal was then amplified with a KWS 30 0 A ampl f er (manufactured by HBM BRD) and stored simultaneously with the EMG signals on the magnetic tape

2 3 *P oc s g of the dat*

Apply ng the data processing system reported elsewhere (Komi and Lehtiö 1973) the stored s gnals were treated in diff rent stages as shown schemat cally in Fig 2 Using the play speed of 15/16 per second the signals were dig tized w th a HP 5610 A analog to d g tal co erter The sampling frequency was 1600 per second The converted signals we e then stored on a digital tape (HP 7970 B Digital Tape Unit) for subsequent analys s with a HP 2116 C (16 K) comput r After the muscle tension had reached the constant level (1 2 s from the beginn ng of contraction) three periods of 625 ms duration were taken for analysis The data were grouped and further treated tatist cally by the same computer and the final results were drawn with a HP 9810 calculator and Hp 91 5 B x–y plotter

The following 3 parameters were nalyzed from the EMG reco dings (1) Integral IEMG (mV/s) ( ) Power spectral dens ty funct on PSDF and (3) Average motor unit potent al AMUP

To compute the PSDF the formulas g en by Bendat and Piersol (1971) were adopted Th s computation started f rst with generation of autocorrelation funct on of the EMG signal for a given time period From this the Fourier transformat on was utilized to obta n PSDF The frequency of the mean power (MPF) was obta ned from the follow ng formula (Kwat y *et al* 1970)

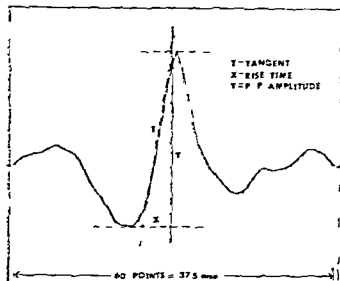


Fig. 3 Schematic drawing showing the method for calculation of rise time and peak to peak amplitude of averaged motor unit potential (AMUP).

f = frequency
f = lower cut off frequency
P(f) = power of a given frequency
f = higher cut off frequency

The values of .4 Hz and 800 Hz were chosen for f and f_{u} respectively.

To relate PSDF to muscle tension the relative (per cent) portion of selected bandwidths (2—5 Hz, 44–96 Hz, 10—135 Hz and 1—800 Hz) were also calculated from the total power density area.

The method developed by Lang *et al.* (1971) was slightly modified for our purpose to compute the ... motor unit potential (AMUP). In short, the computation takes into account all the ... whose amplitudes exceed the desired threshold level. In this experiment the computer was asked to ... 30 sampled points backwards and forwards from the peak. Thus the length of the time-axis was ... ms. The theoretical maximum number of motor unit spikes counted for one AMUP was 50. According to the preliminary trials the threshold level was set to be 120 µV.

AMUP spikes were further analyzed for its peak-to-peak amplitude and rise time. The latter was computed by finding the sign change of the second derivative of the peak rise. A tangent was calculated on this point and the rise time was obtained by taking the difference in x axis between the amplitude peaks and the tangent (Fig. 3).

2.4. Subjects

Six pairs (N = 12) of monozygous twins ranging in age from 13 to 15 years volunteered as subjects. Their physical characteristics are described in Table I. All subjects took part in measurements of the ... but only 5 pairs in data to data comparisons.

The use of twins in the study had no specific purpose for the present problem. The twins served as subjects ... suitable ... also for a present study.

2.5. Measurements

All subjects were carefully trained for the experiment. The actual measurements were taken on different occasions with one day interval. The test session commenced first of 3 maximum voluntary contractions with 3 min recovery intervals between. Then after 5 min rest period the subject was asked to exert the following submaximal contractions: 20, 40, 60 and 80 %. In order to make it easier to exert the desired tension accurately the voltmeter reading the level of tension was placed directly in front of the subjects (see Fig. 1). The recovery periods between contractions was 2 min. Each contraction lasted for 5-10 s.

TABLE I Physical characteristics of subjects.

Twins	Age (y)	Height (cm)	Weight (kg)
1. ♂ A	15	165	47
♂ B	15	164	48
2. ♂ A	14	149	37
♂	14	150	36
3. ♀ A	13	149	45
♀ B	13	151	45
4. ♀ A	13	157	44
♀ B	13	158	46
5. ♂ A	15	18	63
♂ B	15	181	6?
6. ♂ A	14	174	65
♂ B	14	173	65
Mean	14 0	162.8	50 3
±S D	0 9	12 3	10 8

3 Results

Reliability of measurements was investigated by comparing the second and third maximum contractions of the test session The comparison of the results between the different test days was used as estimation of the constancy of measurements Table II summarizes the correlation coefficients for both of these evalutions For muscle tension, IEMG rise time and amplitude of AMUP the reproducibility of measurements was better within the test session than between the different test days The recordings taken from the various band widths of EMG frequency spectrum showed slightly better coefficients in day-to-day comparison but in general these values were rather low Both the reliability and constancy values for MPF were high (r = 0 77–0 88) In maximum contractions the IEMG measure

TABLE II Reliability and constancy coefficients for selected variables

	Level of tension	M rectus femoris	Tensions	IEMG	AMUP Rise time	AMUP Amplitude	AMUP No of spikes	Power spectrum MPF	24–6 Hz	64–96 Hz	104–136 Hz	144–800 Hz
Reliability	100	motor point		88	61	89	.54	.83	63	65	59	90
		middle point	98	91	83	9	71	77	54	55	–4	61
Constancy	100	motor point		73	.34	34	93	.88	81	73	65	89
		middle point	9?	64	7?	56	88	.80	.40	69	76	66
	Tension level range (0 40 60 80 100%)	motor point		86	.59	71	73	.80	8_	7	6_	56
		middle point		80	76	73	.83	76	56	58	9	45

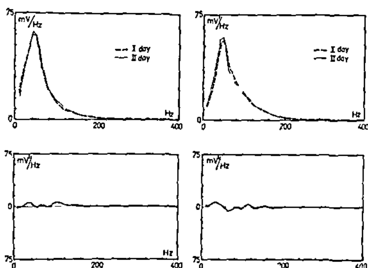


Fig 4 EMG PSDF's (above) recorded from maximum voluntary contractions of the same test situation Left motor point area of m rectus femoris Right mid portion of the same muscle The lower section of the figure denotes the respective power spectral differences between the two contractions Average records of all subjects

ments showed good (r = 0 88–0 91) and satisfactory (r = 0 64–0 73) reproducibility respectively within and between tests The constancy values for IEMG at different tension levels were however better (r = 0 80–0 86) than in maximum contractions The amplitude and rise time of AMUP showed by far the lowest constancy values although the amplitude measurement was rather reproducible in one test session

The average recordings of different parameters for reliability and constancy comparison are shown in *Fig 4 5 and 6 This* group treatment revealed similar *PSDF's both in the*

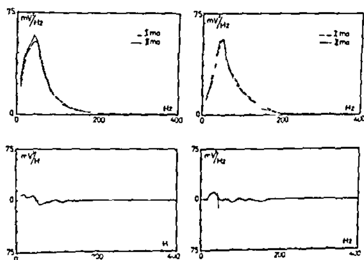


Fig. 5 Comparison of PSDF in maximum contraction between separate test days For explanation see Fig 4

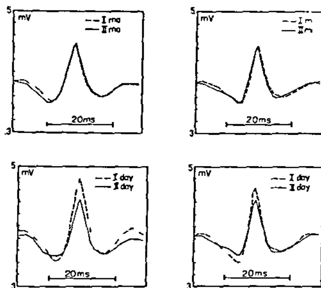


Fig. 6 Graphed AMUPs in within test (above) and day to-day (below) comparison of maximum contractions Left motor point area Right mid portion of m. rectus femoris Average records of all subjects

same test session (Fig 4) and between the two test days (Fig 5) The lower portions of Fig 4 and 5 also show that the mean curves differed only slightly from each other The mean AMUPs were superimposed when the two maximum contractions of the same test were compared They however differed distinctly when the comparison was made on day to-day basis (Fig 6)

Discussion

When considering the repeatability of EMG measurements several factors should be taken into account These include the types of electrodes used (Komi and Buskirk 1970 Jonsson and Komi 1973) electrode contact area (Goddes *et al* 1967) electrode placement (Lippold 1967) and source and amplifier input impedance (DeVries 1966 Schanne and Chaffin 1970 Rau 1973) Most of these factors have been discussed in connection with the global type of EMG recordings *e g* IEMG but their applicability to somewhat finer EMG analysis such as the different signal characteristics employed in the present study are naturally of similar importance What ever the parameters are they should all come from the same EMG activity In the present study the EMG s were first digitized and the different analysis were performed exactly from the same time period

Perhaps the most important factor in the repeatability problem of the EMG recordings is the control over the amount and quality of the neural input to the muscle The whole muscle EMG activity is a very complex biological phenomenon from which it is not easy to differentiate the contributions of different signal sources such as reflex activity and the inputs from the higher motor centers Thus it is natural to expect the input signal vary considerably from time to time both in quantity and quality Maximum voluntary contraction is an example of a condition in which one can expect considerable variations Yet it may be an acceptable way to standardize the test situations in human experiments The negligence

in the control over the direction of movement may add to the variations in the EMG activity also during maximum effort Type and velocity of contraction (*e g* Bigland and Lippold 1954 Komi 1973) and muscle length (*e g* Håkansson 1957) are also factors which have been shown to influence the EMG recordings

The observed reliability of IEMG measurements (r = 0 88–0 91) are within the values reported earlier in the literature (Komi and Buskirk 1970 Kramer *et al* 1972) and can thus be regarded as acceptable The constancy coefficients of IEMG (r = 0 64–0 86) were also satisfactory and in accordance with those earlier reports It should be emphasized that part of the lowered correlation coefficients can be accounted for by the differences in maximum contractions The repeatability coefficients for muscle force were 0 98 and 0 92 respectively within and between tests

Both the reliability (r = 0 77–0 83) and constancy (r = 0 76–0 88) coefficients of MPF tend to indicate that this parameter can be considered suitable for studies in which the measurements are repeated over longer periods of time However the poorer repeatability coefficients observed for the different bandwidths of the EMG spectrum may point out that their applicability as EMG parameters for followup experiments is slightly weaker than that of a more representative type variable MPF

Rise time amplitude and the number of composite spikes of AMUP showed also satisfactory reliability However the constancy coefficients were relatively poor for AMUP rise time and amplitude In day to-day comparison the group AMUP s were not superimposed as was the case within one test situation and they differed more from each other in the recordings taken from the motor point area (Fig 6 lower left) Similarly the constancy coefficients for rise time and amplitude were lower for recordings from the motor int area This may point out the difficulties and possible errors in placing the electrodes ctly on the same position on the muscle Despite careful marking of the skin for electrode tes one can still not be quite confident on the exactness of the replacement The electrodes on the upper third of the rectus femoris muscle were placed over the motor point area It is reasonable to expect the EMG amplitude be highest within that region This was also the case when observing the AMUP curves of the two measurement sites (Fig 6) According to the findings of Gydikov et al (1972) on the amplitude and form changes of the EMG potential along the motor line it is quite possible that the differences in electrode replacement may have had a greater effect on the AMUP amplitude changes in the recordings obtained from the second electrode pair which was located at a considerable distance from the motor point (see the insert in Fig 1)

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Acta physiol scand 1975 93 540–547

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Biogenic Amines in Carotid Body of Adult and Infant Rats — a Gas Chromatographic–Mass Spectrometric Assay

By

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Received 5 November 1974

Abstract

HELLSTRÖM S and S H KOSLOW *Biogenic amines in carotid body of adult and infant rats—a gas chromatographic–mass spectrometric assay* Acta physiol scand 1975 93 540–547

A gas chromatographic mass spectrometric method was used for the determination of biogenic amines in carotid body of adult and 10 days old rats The method is ideally suited for this measurement since only small amounts of tissue were available (dry weight carotid body adult 8 3 μg infant 5 6 μg) In adult carotid body large amounts of dopamine (1 950 pmol/mg protein) and norepinephrine (1 140 pmol mg protein) were found together with a comparatively small concentration of serotonin (505 pmol mg protein) The carotid bodies of infant rats contained 1 065 pmol dopamine/mg protein and 410 pmol norepinephrine/mg protein Epinephrine could not be detected Surgical sympathetic denervation and chemical sympathectomy (6-hydroxydopamine) of adult carotid bodies did not significantly change the catecholamine content as compared to the controls Reserpine depleted the catecholamines dosedependently Administration of L Dopa and pargyline (a monoamineoxidaseinhibitor) drastically increased the concentration of catecholamines Treatment with a dopamine β hydroxylase inhibitor resulted in a decreased amount of norepinephrine without a simultaneous increase of dopamine This may indicate that certain storage sites in this tissue may store dopamine while in other sites dopamine is a precursor of norepinephrine Probably most of the dopamine and norepinephrine are stored in different cells

The occurrence of biogenic amines in carotid body cells of several species (e g cat rabbit rat) has been shown by the use of fluorescence histochemical methods (for ref see Biscoe 1971 Bock 1973 Möllman *et al* 1972 a) Moreover fluorometry has revealed quantitative estimates of norepinephrine dopamine epinephrine and serotonin (for ref see Bock 1973 Lishajko 1970 Möllman *et al* 1972 a) The amount of different amines stored in the carotid body is however varying The variation has been suggested to depend on differences between species as well as between individual animals (Zapata *et al* 1969) Moreover technical problems related to the minute size of the carotid body of most animals investigated have hampered the chemical analysis Thus the quantitation of the amine content in a single carotid body from adult and infant rats has been difficult if at all possible With the

mass fragmentography assay of catecholamines and serotonin (Cattabeni Koslow ~ Costa 1972 Koslow Cattabeni and Costa 1972) which allows detection of at least 2υ 10^{-15} mol it is possible to overcome the problems caused by a limited amount of tissue available

In this present study the mass fragmentographic methods were used to determine the catecholamine and serotonin content of carotid body from normal adult and infant rats

Furthermore the chemical analysis of biogenic amines in the carotid body were made after surgical excision of the sympathetic ganglioglomerular nerves and after treatment with drugs known to inhibit certain pathways of the monoamine metabolism

It will be shown that the carotid body of the rat contains large amounts of dopamine norepinephrine and serotonin the stores of these amines are depleted by reserpine and may be present in different cells

Material and Methods

Adult rats

The chemical analyses were carried out on carotid bodies from male Sprague Dawley rats (? months old) weight about 175 g All rats were killed by a blow on the head the carotid bodies were thoroughly cleaned from surrounding connective tissue and rap dly removed for the chemical analyses of the biogenic amines The dry weight of one carotid body was estimated to be $8.3 \pm 0.89\ \mu g$ (mean ± S E n = 8) The dopamine norepinephrine and epinephrine content were determined in all rats the serotonin only in rats not exposed to surgical or drug treatment

Sympathetic dener ation of ca otid bod The ganglioglomerular nerves wh ch connect the superior cervical ganglion to the carotid body were surgically exc sed unilaterally on 10 anesthetized animals under stereo microscope control 20 days after the operation the rats were killed and the amine content of the sympathetically denervated carotid body analyzed The carotid body of the opposite side was removed and used as control

Chemi al sympathectomy (Thoenen and Tranzer 1968) 6 rats were treated with 6 hydroxydopamine (6-OH DA) for ₂0 days 6 OH DA was injected at 3 times ?0 16 and 1 days before the rats were killed at a dose of 100 mg/kg b wt through a tail vein

Rese pine The rats were divided into 3 groups and reserp ne was administered i v through a ta l vein One group of 4 rats was given 0 1 mg reserpine/kg b wt another group of 4 rats was given 0 5 mg/kg and the remaining group of 3 rats 1 0 mg/kg. 2 h after the injection the rats were k lled

L-Dopa (L d hyd oxy ph nylalan n) 4 rats were given 100 mg L Dop /kg b wt intraperitoneally 3 times at 15 9 and 3 h before sacrifice

Pa gyl ne 4 rats were given pargyl ne 100 mg kg i v h before the rats were killed and the carot d body removed

DDC (D ethyldith o a bamate) 3 rats were gi en 00 mg DDC/kg killed 2 h later and the carotid body d ssected out and processed for chemical an lysis

All d g with the exception of reserpine were d ssol ed in 0 9 saline The reserpine was d ssolved in distilled water after addit on of a few drops of acet c acid

Contr l 5 rats g en s l ne were used as control

I f nt rats

The chem cal analysis was performed on carotid bod es from 10 days old Sprague Dawley rats weight about 25 g Th infant rats were k lled by a blow on the head and the carotid bodies dissected out in a s m lar way to that of the carotid bod es from adult rats The dry weight of one carotid body was estimated to be 5.6 ± 0.38 / g (mea ± S E n = 9) Th dopam ne norepinephrine and epinephrineco tent were determined

Gas ch omatog phy–m s sp ct om t y (GC MS) a al sis

Cat cholam e a ay Norep neph ne (NE) epinephrine (E) a d dopamine (DA) were determined by mass fragmentography (Koslow Cattabe i and Costa 197₂ Koslow 1973) In brief the carotid bodies

were homogenized in 100 µl of a 50 mM solution of ascorbic acid in 0 1 N formic acid After centrifugation the supernatant was transferred to small glass vials

Two internal standards α methyl norepinephrine (α MNE) and α methyl dopamine (α MDA) were added to the supernatant The content of the vial was dried under a stream of nitrogen The catechol amines were then acetylated by addition of 100 µl pentafluoropropionic anhydride (PFPA) (Pierce Chemicals) and 20 µl ethylacetate and heating at 60 C for 30 min Shortly before the analysis the excess PFPA was evaporated under a stream of nitrogen and the residue redissolved in 10 µl ethylacetate 2 µl of this solution were injected into the GC MS (LKB 9000)

GC conditions A 9 feet glass column 2 mm (i d) packed with 3 SE 54 on chromosorb G Flash heater temperature 250 C oven temperature 200 C helium flow 30 ml/min Under these conditions the catecholamine derivatives had the following GC retention times (sec) α MNE 100 NE 110 E 150 α MDA 195 DA 265

MS conditions Molecular separator 250 C Ion source 270 C trap current 60 µA electron energy 80 eV and electron multiplier 3 5 kV The mass to charge (m/e) ratio focused for quantitation of the acetylated catecholamines were α MNE 190 NE 176 E 190 α MDA 190 DA 176 The fragments monitored for identification were m/e 577 (12) and m/e 549 (7) for NE and m/e 428 (100) and m/e 415 (5) for DA (Costa et al 1972) The correct fragment ratios of 1 7 and 20 were obtained for carotid body NE and DA respectively

Serotonin assay Serotonin (5 HT) was determined by mass fragmentography (Cattabeni et al 197) The carotid bodies were homogenized in 100 µl of an ice cold solution which consisted of 50 mM ascorbic acid in formic acid/acetone (5 95) After centrifugation the supernatant was transferred to small vials An internal standard α methyl serotonin (α M5HT) was added to the supernatant and the vial processed similarly to the procedure for the catecholamine assay The indolamine derivatives had the following GC retention times on 3 OV 17 on Gas ChromQ α M5HT 150 s 5HT 200 s The mass to charge (m e) ratio focused for quantitation of the acetylated indolamines were α M5HT 438 5HT 438 The fragments monitored for the identification of 5HT were m/e 438 (44) and m/e 451 (100) Authentic 5HT and the carotid body extract both gave the fragment ratio of 2 3 at the GC retention time of 5HT (Koslow and Green 1973)

Presentation of data

The quantitative data presented represents the amine content in picomoles per 2 carotid bodies (from one the same rat exception in the denervation experiments from 2 rats) as mean ± S E The control values are also presented as picomoles amine per mg protein The amount of protein was determined according to Lowry et al (1951) The statistical significance of the results was determined by Student s t test (two tailed)

Results

Adult controls (Fig 1) A pair of carotid bodies contained an average of 28 9 pmol DA (1 953 pmol DA/mg protein) and 16 9 pmol NE (1 142 pmol NE/mg protein) E pinephrine could not be detected in spite of the sensitivity of our method of at least 0 1 pmol Serotonin was present in concentration of 7 5 pmol per pair of carotid bodies (506 pmol 5HT/mg protein)

Infant rats (Fig 1) A pair of carotid bodies contained on an average 11 1 pmol DA (1 067 pmol DA/mg protein) and 4 3 pmol NE (410 pmol NE/mg protein) E pinephrine was not detected The amounts of dopamine and norepinephrine per mg protein was less in the carotid body from the infant rat than in the carotid body from the adult rat

Denervation (Fig 2) Although the values indicate a slight decrease of dopamine (24 6 ± 5 16 pmol) and a tendency of an increase in the norepinephrine concentration (22 3 ± 2 36 pmol) they did not differ significantly as compared to the control values

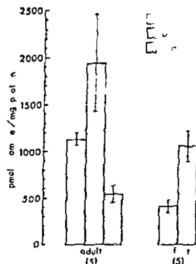


Fig. 1 Concentration of biogenic amines in carotid body of adult and infant rats The amount of serotonin (5 HT) was determined in the adult only The amine concentrations are given as mean ±S E. pmoles per mg protein Number of rats in parenthesis

Chemical sympathectomy (Fig 2) The values (DA 27 2±4 66 pmol NE 15 5±2 11 pmol) did not differ significantly from the controls

Reserpine (Table I) The amount of dopamine and norepinephrine was significantly decreased after reserpine treatment When treated with 1 0 mg/kg more than 96% of the

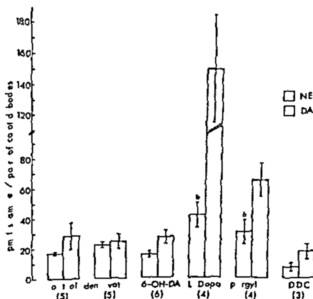


Fig 2 The concentrations of norepinephrine and dopamine of carotid bodies from adult rats after sympathetic denervation different drug treatments and controls (for details see Material and Methods) The concentrations of catecholamines are given as mean ±S E. pmoles per pair of carotid bodies. Number of rats in parenthesis (a) p<0 05 (b) p<0 0 ( ) p<0 01 when compared to the mean of the controls.

TABLE I Reserpine depletion of norepinephrine (NE) and dopamine (DA) in the carotid body of adult rats. Number of animals in parenthesis

Reserpine (mg/kg i v)	NE	DA	NE	DA
	pmol pair of cb (mean ± S E)		% depletion	
0	16 9 ± 1.03	28.9 ± 7 85 (5)	0	0
0 1	[a]8 83 ± 3 04	16.5 ± 4 11 (4)	47 7	42.9
0 5	[a]3 98 ± 0 663	[a]3 16 ± 0 818 (4)	76 4	89 1
1 0	[a]0.576	[a]1 11 ± 0 469 (3)	96 6	96.2

[a] $p < 0\ 05$ when compared to the mean of carotid bodies from untreated rats.

dopamine and norepinephrine-content was depleted The depletion was shown to be dose dependent

L-Dopa (Fig. 2) When administering L Dopa in the doses described there was a 5-fold increase of the amount of dopamine (150 ± 35 5 pmol) and a doubling of the norepinephrine (41 5 ± 8.5 pmol) Epinephrine was not detected.

Pargyline (Fig. 2) This monoamine oxidase inhibitor significantly increased the amount of norepinephrine (30.2 ± 7 9 pmol) as well as dopamine (64 7 ± 11 2 pmol)

DDC (Fig. 2) This drug significantly lowered the concentration of norepinephrine (7 0 ± pmol) The amount of dopamine (17 6 ± 4 76 pmol) was not significantly changed compared to the controls

Discussion

This study showed that the gas chromatographic mass spectrometric method for identification and quantitation of biogenic amines (Koslow *et al* 1972) could be applied to the identification and measurement of the amines of the carotid body of adult and infant rats even though only small amounts of the tissue were available The amines found were primarily DA and NE and to a minor extent serotonin Dopamine dominates in accordance with earlier chemical investigations of carotid body from rabbit and cat (Dearnaley Fillenz and Woods 1968 Chiocchio *et al* 1971 b Zapata *et al* 1969) The relatively smaller amount of amines in the infant carotid body does not correlate to the fluorescence microscopical findings by Hervonen *et al* (1972) These authors describe a more intense catecholamine fluorescence in the infant rat carotid body compared to the adult one The cells of the infant rat carotid body may be smaller thus the fluorescent material is more compact giving the impression by fluorescence microscopy of a greater catecholamine concentration

In carotid bodies of infant and adult rats epinephrine was not detectable The serotonin content of the adult carotid body was lower than that of the catecholamines Möllman *et al* (1972 a, b) using microspectrofluorometry reported that serotonin is present in rabbit

carotid body and that this fluorescence disappears after parachlorophenylalar ι treatment In contrast Chiccchio King Angelakos (1971 a) found very fev e fluorescing cells in cat carotid body Furthermore they did not observe any altera ic this fluorescence after PCPA treatment Bock (1973) postulated that the seroto ır tected by fluorescence microscopy in mouse and rat carotid body might be localized the mast cells which occur in the interstitial tissue between islands of carotid body Type I (glomus chemoreceptor) cells The localization of 5 HT to the Type I cells as postulated for the human carotid body by Hamberger Ritzen and Wersall (1966) however cannot be excluded

The sectioning of ganglioglomerular nerves did not change the catecholamine content Sympathectomy has been shown to decrease the amine content in tissues innervated by noradrenergic nerves (Euler and Purkhold 1951) In cats Zapata *et al* (1969) sectioned the ganglioglomerular nerves and found that the catecholamine content of carotid body was unchanged by denervation These results favour the hypothesis that the amines contained in the carotid body are stored in the Type I cells since the level of catecholamines appears to be independent from sympathetic innervation Perhaps these nerves are not abundant and may not even innervate the catecholamine stores but impinge upon the cells of the vascular bed (Zapata *et al* 1969)

The fact that 6-OH DA did not cause any change of the catecholamine content was rather suprising as Lassmann and Bock (1972) reported a strongly diminished fluorescence and a decrease in number of the dense cored vesicles of the Type I cells after a similar 6-OH DA treatment One possible explanation of the diverging results might be that the effects of 6-OH DA on adult carotid body cells are rapidly reversible That would mean that the amine stores of the carotid bodies from the rats in the present study were restored as the rats were killed 12 days after the last injection A second possible explanation is that 6-OH DA leaves the adult carotid body cells unaffected In this respect the cells of carotid body may resemble chromaffin cells of adrenal medulla (for ref see Thoenen and Tranzer 1973)

The amines of carotid body Type I cells are most probably localized in their granulated vesicles (for ref see Bock 1973) Several morphological investigations have dealt with problems concerning whether or not reserpine depletes the content of the granulated vesicles Chen Yates and Duncan (1969) reported that reserpine treatment resulted in a decreased density of the vesicle content Further Bock and Lassman (1973) reported a decrease in number of the dense cored vesicles after reserpine treatment The present study established that reserpine in a dose dependent fashion depletes the catecholamines They disappeared completely with increasing doses of reserpine A comparison of this data with the above mentioned morphological studies (Chen Yates and Duncan 1969 Böck and Lassmann 1973) where the authors in spite of their findings still observe a black content of the vesicles one might conclude that the black granules do not as a whole represent the amine stored but more probably a matrix connected with the amine

The L Dopa treatment resulted in massive increase of dopamine and norepinephrine The rate limiting step of the catecholamine synthesis the enzyme tyrosine hydroxylase is by passed and the catecholamines are synthesized in proportion to the amount of

administered. The L-Dopa findings are in accordance with a recent quantitative ultrastructural study (Hellström 1975 b) where an identical L-Dopa treatment was shown to increase the volume density and the size of the granulated vesicles The enlargement of the size increases the volume of the spherically shaped vesicle 2 times which in turn makes it possible for the cells to store the very large amount of catecholamines synthesized.

The monoamine oxidase inhibitor pargyline as expected increases the amount of dopamine and norepinephrine These observations correlate well with earlier findings with the fluorescence microscopical technique (Chiocchio *et al* 1971 a) The amount of amines seem to double within 2 h when monoamineoxidase is blocked This would indicate that the half life of dopamine and norepinephrine might be at least 1 h This experiment as well as the L-Dopa experiment showed that the dopamine increased proportionally faster than norepinephrine thus indicating that dopamine has a faster turnover rate than norepinephrine

The dopamine β-hydroxylase inhibitor DDC was administered to study if dopamine plays some other role in addition to its role as the precursor of norepinephrine If dopamine were only a precursor for norepinephrine one would expect a decrease in norepinephrine and an increase in dopamine concentration The results showed a decrease in norepinephrine without an increase in dopamine This observation indicates that only part of the dopamine present serves as a precursor Most probably norepinephrine and dopamine are stored in different cells These different types of cells might well correlate to the two kinds of Type I cells which are distinguished, morphometrically by the diameters of the granulated vesicles (Hellström 1975 a) Since the most frequent cell type found contain a large number of big vesicles these large vesicle cells might store dopamine which is the predominant amine the carotid body of rats Perhaps the small vesicle cells might store norepinephrine

We are now continuing the experiments to test whether this working hypothesis can be substituted using pharmacological agents which selectively affect the dopamine or the norepinephrine content of the rat carotid body Our final aim being that of elucidating what role these types of cells and the catecholamines they store might play in the function of the carotid body

We wish to express our gratitude to Dr E. Costa, in whose laboratory this work was done for generous support and critical reading of the manuscript We thank Dr J Sehlin for determination of carotid body dry weight

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Acta physiol scand 1975 93 548–552

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The Distribution of Sodium in Aortic Walls from Spontaneously Hypertensive and Normotensive Rats

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Received 6 November 1974

Abstract

JONSSON O Y LUNDGREN and G WENNERGREN *The distribution of sodium in aortic walls from spontaneously hypertensive and normotensive rats* Acta physiol scand 1975 93 548–552

The contents of exchangeable sodium bound sodium and total water and the extracellular space of thoracic aortas from normotensive and spontaneously hypertensive rats were measured The aortas from the hypertensive rats contained more sodium than those from the normotensive animals while the total water content and extracellular space in the two groups were the same The capacity to bind sodium in an osmotically form was greater in the aortas from the hypertensives than in those from the normotensives difference in binding capacity was of the same order of magnitude as the difference in sodium content indicating that the excess sodium in the thoracic aortas from the hypertensive rats was osmotically inactive and thus unable to cause water logging

Hemodynamic studies in man and in spontaneously hypertensive rats (SHR) strongly suggest that the increased resistance in established primary hypertension is mainly the result of a pressure induced structural adaptation of the precapillary resistance vessels (*cf* Folkow *et al* 1973 1974) This structural autoregulation in nature closely related to the well known left ventricular hypertrophy in hypertension consists of a rapidly established increase in media thickness encroaching upon the lumen even at maximal dilatation The consequent enhancement in wall/lumen ratio will exaggerate the reactivity of the precapillary resistance vessels without necessitating an increased sensitivity of the vascular smooth muscle cells Such a positive feedback interaction between vessel design and smooth muscle function implies a vicious circle escalating the pressure Since related structural changes in larger arteries contribute to the baroreceptor resetting hence adjusting also the reflex pressure control to higher levels the vicious circle will be potentiated The hemodynamically important changes in vessel design have also been histologically analyzed and fully verified in extensive studies of systemic vascular beds in hypertension (Furuyama 1962, Short 1966 Suwa and Takahashi 1971)

Tobian and Binion (1952) found a certain degree of *wall edema* associated with an

increased sodium content in the renal arteries from patients who died in hypertension. They suggested that such a water logging if present also in the resistance vessels could explain the increased resistance to flow found in hypertensive subjects. An increased sodium content has also been observed in the aortas from SHR with established hypertension while no such change was found in younger SHR with labile hypertension (… Kikuchi and Aramaki 1970). Thus the early structural adaptation of the precapillary … in primary hypertension might in later phases be superimposed by a certain degree of water logging. However in the mentioned study only the *total* sodium content of the aortic wall was determined without any distinction between osmotically active and inactive sodium. The present study was performed to explore whether the reported increase in aortic sodium content could be bound *e g* to an increased amount of ground substance being then osmotically inactive and consequently unable to produce water logging. This would help to settle the question whether water logging in the resistance vessels proper might contribute to the raised resistance in established primary hypertension.

Methods

Thoracic aorta preparations from 41 spontaneously hypertensive rats (SHR) (*cf* Okamoto 1969) of both sexes in the phase of well established hypertension (30–40 weeks old) and from 40 otherwise matched normotensive control rats (NCR) were used. The resting arterial blood pressures were recorded in the awake animals by means of intra arterial measurements in the caudal arteries.

Preparation of vessel After killing the animals by a blow on the neck the abdomen and thorax were immediately opened and the thoracic aorta carefully dissected free. Fat and connective tissue surrounding the vessel were removed under dissection microscope. In order to reduce diffusion distances during incubation and to facilitate the blotting procedure the vessel was cut open longitudinally. Uniform size of the preparations was obtained by dividing the artery into 3–4 smaller pieces. The preparations were allowed to accommodate for 30 min at 37 C in Krebs solution (NaCl 122, KCl 4 73, $NaHCO_3$ 15 5, KH_2PO_4 1 19, $MgCl_2$ 1 19, $CaCl_2$ 2 49 and glucose 11 5 mmoles/l) aerated with a gas mixture consisting of 96 … O_2 and 4 … CO_2. After this preincubation some of the vessels were immediately used to determine the distribution spaces of … Na and ^{14}C sucrose as well as the ratio dry weight wet weight. These vessels will later be referred to as fresh vessel preparations. The other preparations were slowly frozen down to − … 0 C in Krebs solution stored at this temperature for at least 48 h and then slowly thawed in order to destroy the cell membranes thus obtaining a homogeneous fluid phase for ionic distribution. After a second preincubation of these frozen and thawed vessel preparations the distribution space of … Na as well as the ratio dry weight–wet weight were determined.

Distribution of … Na and … C sucrose The procedures to determine the uptake of radioactive tracers in the vessel preparations were the same as earlier used for the rat portal vein (Arvill *et al* 1969) and will here only be presented in brief. After preincubation the preparations were transferred to flasks containing 2 ml Krebs solution to which … Na had been added (specific activity 14 mCi mmol at the beginning of the experiment). The flasks were aerated with 4 … CO_2 in O_2 for 30 sec every 30 min. After 1 0 min incubation the preparations were gently blotted between 2 pieces of filterpaper, weighed on a Cahn electrobalance and homogenized in 10 … tri chloro-acetic acid (TCA). This procedure was performed in a strictly standardized manner. The radioactivity of the muscle extracts and of the incubation media were determined with a Packard Tri–Carb liquid scintillation counter (for details see Arvill *et al* 1969). Double samples were always analyzed.

The extracellular space in fresh vessel preparations from the thoracic aorta, under the above mentioned experimental conditions was determined by measuring the 30 min uptake of … C sucrose (specific activity 3 3 mCi mmol) after 1?0 min preincubation exposing the preparations to exactly the same incubation conditions as those used in the … Na uptake experiments. In preliminary experiments it was found that 30 min of incubation in the … C sucrose medium was sufficient for the tracer substance to equilibrate in the vessel specimens. The sodium and sucrose *spaces* were calculated from the distribution of radioactivity between the tissue and the medium and expressed in ml 100 g wet tissue weight.

TABLE I Illustrating values obtained with fresh vessel preparations of thoracic aortas from NCR and SHR Mean ± S E. The number of animals used in each group is given within brackets

	Dry weight ml/100 g wet weight	^{14}C sucrose space ml/100 g wet weight	^{24}Na space ml/100 g wet weight	Bound + intracellular Na ml/100 g wet weight
NCR	32 8 ± 1 0 (7)	49 7 ± 0 6 (8)	57 2 ± 0 6 (9)	7 5
	p<0 8	p<0 3	p<0 006	
SHR	32.5 ± 1 0 (8)	48 5 ± 0 7 (8)	60 3 ± 0 9 (9)	11 8

Dry weight Dry weight estimations were performed both on fresh vessel preparations and on frozen and thawed preparations according to the same time schedule as used in the ^{24}Na uptake experiments described above When the vessel preparations had been preincubated for 30 min they were transferred to flasks containing 2 ml Krebs solution. After 120 min in this solution they were blotted and weighed (wet weight) The preparations were then dried (for at least 24 h) to constant weight in a vacuum oven at 100 C and reweighed (dry weights) The total water content was calculated as the difference between wet weight and dry weight The dry weights are expressed in gram per 100 g wet weight

In order to find out whether the freezing and thawing procedure altered the wet weight of the preparations determinations of the wet weight were made both before and after that procedure

Fractions of bound ions When the space calculated for an ion in the frozen and thawed muscle preparation exceeded the total water content of the vessels the excess was regarded to represent a fraction of bound ions The bound ion fractions are expressed in ml/100 g wet tissue weight

Results

The directly measured mean arterial pressures were 103 ± 2 mm Hg in NCR and 155 ± 3 in SHR respectively ($p < 0\,001$) The mean values for dry weights ^{14}C sucrose and ^{24}Na spaces of the fresh vessel preparations from the thoracic aortas are given in Table I The amounts of bound sodium plus intracellular osmotically active sodium which cannot be separated with the present technique were estimated from the differences between the ^{24}Na and the ^{14}C sucrose spaces The exchangeable sodium content expressed in meq/kg wet weight which was calculated by multiplying the ^{24}Na space with the sodium concentration in the incubation solution amounted to 78 3 and 82 6 meq/kg wet weight for NCR and SHR respectively The corresponding values for bound + intracellular Na amounted to 10 3 meq/kg wet weight for NCR and to 16 2 meq/kg wet weight for SHR

The data obtained with the frozen and thawed preparations are given in Table II The wet weight of these specimens was reduced approximately 10 per cent The aim of the freezing thawing procedure was to destroy the cell membranes thus obtaining a homogeneous tissue fluid phase with regard to ionic distribution It has previously been found that such a treatment enables sucrose generally supposed to diffuse only in the extracellular space to equilibrate in the total fluid phase of vascular smooth muscle (Haljamae *et al* 1969) In the present study the fraction of the ^{24}Na space of the frozen thawed vessel preparations that exceeded the total water space was considered to represent the Na binding capacity of the arterial walls Thus this binding capacity was estimated from the difference between the ^{24}Na space and the total water content

After correction for the change in wet weight the difference in binding capacity between

TABLE II. Illustrating values obtained with frozen and thawed vessel preparations of thoracic aortas fr NCR and SHR. Mean ± S.E. The number of animals used in each group is given within bracke s

	$\frac{\text{Wet weight frozen specimen}}{\text{Wet weight fresh specimen}}$ 100	Dry weight ml 100 g wet weight	[?]Na space ml 100 g wet weight	Na binding capacity ml 100 g wet weight
NCR	91 7 ± 1.3 (9)	33 9 ± 0 8 (9)	79 8 ± 1 6 (7)	13 7
	$p < 0.1$	$p < 0.7$	$p < 0.01$	
SHR	89 0 ± 0 9 (9)	34.2 ± 0 [illegible] (9)	85.2 ± [illegible].7 (7)	19 4

esh vessel preparations from SHR and NCR corresponds to a difference in bound amounts sodium of 6 4 meq/kg wet weight, provided the excess binding sites are equilibrated with the extracellular sodium concentration

Discussion

The relative contributions of functional and structural elements to the raised resistance in primary hypertension have been much debated However it now seems increasingly clear that a rapidly established structural autoregulation of the precapillary resistance vessels (see Folkow *et al* 1973 1974) is of crucial importance for the constitution of a truly hypertensive state Thus the increased wall thickness of the resistance vessels seems to be mainly due to a media hypertrophy Another possibility of great interest is that of a water logging of arterial walls (*cf* Tobian 1972) which, if present also in the resistance vessels proper would raise resistance and pressure

An increased arterial sodium content has, in fact been found in SHR with established hypertension (Nagaoka Kikuchi and Aramaki 1970) but no distinction was then made between osmotically active sodium and inactive (bound) sodium The present experiments were performed in order to investigate whether the mentioned sodium increase really represented an osmotically active fraction which would be a prerequisite for causing true water logging. It was however found that the water content and the extracellular space per unit wall weight were *not* significantly different in SHR and normotensive control rats (NCR) excluding the presence of any interstitial water logging in the arterial walls of SHR with established hypertension

The amounts of exchangeable sodium as well as the binding capacity of sodium were however greater in the SHR aortas To calculate the amounts of bound sodium in the fresh vessel preparations it is necessary to know whether the binding sites are equilibrated with the extra or intracellular sodium concentrations Experimental studies by Jonsson (1971) indicate that cations are sequestered both extra and intracellularly However since the sequestration of cations occurs mainly in the ground substance of the vascular wall (Headings Rondell and Bohr 1960) and since the amount of ground substance is considerably increased in hypertension (Crane 1962) it seems justified to assume that all additional sodium binding sites in the arteries from SHR are equilibrated with the extracellular sodium concentration If so the difference in exchangeable sodium content in the aortas from NCR and SHR can be fully ascribed to a greater amount of sequestered osmotically inactive sodium in the latter group

It thus follows that there is no evidence of either any interstitial or any intracellular water logging in the aortic walls of SHR with established hypertension Essentially all the sodium increase present may be ascribed to the bound sodium fraction which is presumably linked to the ground substance known to become increased in arterial walls along with hypertension This is in line with the fact that the early hypertrophic increase in wall/lumen ratio (see Introduction) is gradually followed by collagen formation and other interstitial changes in the vascular walls (*cf* Wolinsky 1972) whereby the fraction of bound sodium would also increase This does not deny that some water logging might become superimposed in a later phase when *e g* renal complications imply an addition of hormonal and electrolyte disturbances A modest element of aortic water logging can thus be traced in longstanding renal hypertension in rats (*e g* Tobian 1972 Lundgren 1974) but the hemodynamic contribution of such an element is likely to be far weaker and far later in appearance than that caused by the early adaptive increase in media thickness of the resistance vessels

This work was supported by grants from the Swedish Medical Research Council (No B74 14X 16 11A) from the Swedish National Association against Heart and Chest Diseases and from the Faculty of Medicine University of Göteborg

Thanks are due to Mrs Siv Samuelsson for skilful technical assistance

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Acta physiol scand 1975 93 553-559
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Inefficiency of Isoprenaline to Induce Drinking in the Goat

By

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Received 8 November 1974

Abstract

OLSSON K and M RUNDGREN *Inefficiency of isoprenaline to induce drinking in the goat* Acta physiol scand 1975 *93* 553-559

Isoprenaline which acts as a potent dipsogen in water-satiated rats and dog did not elicit wate intake when infused intravenously at 0 1 or 0 3 μg/kg min^{-1} in non hydrated goats E en the low dose of the drug caused a marked reduction of parotid sali ary flow The possibility is discus ed that reduced sal ary secretion might be the particular effect wh ch makes isoprenaline dipsoge ic in prandially dri king species The intravenous infusion of isoprenal ne at the high dose le el caused an inhib t on of the w t r diuresis of hydrated goats, concomitant with reduced renal Na+ e cretion and a marked susta ned fall in the arterial blood pressu e S gnif cant amounts of ADH were reco red from the urine secreted duri ig the antid ures s Th s ADH release was apparently not due to central β-adrenergic stimulation since no inhibition of the water d uresis was observed during intra entricular infusions of isoprenal ne Rather the ADH release appears to have been secondary to the isoprenaline induced fall in arterial blood pressure

Both a marked dipsogenic and an antidiuretic effect of β adrenergic agonists have been demonstrated in two mammalian species the rat (Zamboni and Siro-Brigiani 1966 Lehr Mallow and Krukowski 1967) and the dog (Fitzsimons and Szczepanska Sadowska 1974) From available data it appears that the mechanisms behind this β-adrenergic effect on the water turnover differ in the two species

Suggestive evidence has been presented that the ultimate cause of β adrenergic drinking in the rat is an activation of the renin angiotensin system previously shown to induce drinking in this species (*cf* FITZSIMONS 1972) Hence the administration of β adrenergic agonists to rats causes an elevation of plasma renin activity (Peskar *et al* 1970) and rats no longer drink in response to β adrenergic agonists after nephrectomy (Houpt and Epstein 1971)

An activation of the renin angiotensin system appears to be of no or of only minor importance for the dipsogenic response to β adrenergic agonists in the dog Angiotensin II is relatively ineffective in eliciting drinking in this species (Kozlowski Drzewiecki and Żurawski 1972) and nephrectomy does not significantly reduce drinking in response to isoprenaline which suggests that non hormonal mechanisms are much more important for the dipsogenic response than in the rat (Fitzsimons and Szczepańska Sadowska 1974) The dog is a prandial drinker (Wolf 1958) but it remains to be studied whether oropharyngeal factors contribute to its isoprenaline induced drinking

The apparent difference between the mechanisms behind β-adrenergic drinking in the rat and in the dog has provided the incitament for this study of the effects of isoprenaline on the water balance and the salivary secretion in the goat

Methods

Animals 10 adult female goats (b wt. 35–40 kg) were used The animals were routinely kept in metabolism cages where all experiments were conducted They were fed chopped hay every morning at 8 o clock and the experiments were not started until at least 2 h later They had free access to water at a temperature of 20 ± 1 C and were maintained in positive sodium balance by obtaining 6 g of NaCl in 300 g of commercial grain mix each afternoon

Administration of isoprenaline Isoprenaline dissolved in isotonic saline was infused intravenously via a polyethylene cannula introduced into the jugular vein The cannula was connected with a perfusion apparatus via a polyethylene tubing. In most experiments isoprenaline hydrochloride (Isuprel Winthrop) was used Isoprenaline sulphate (Aludrin Boehringer Sohn) was infused in a few experiments The dose of isoprenaline was 0 1 or 0 3 μg/kg min $^{-1}$ and the infusion periods were 50 or 60 min

Infusions (20 μl min) into the cerebrospinal fluid (CSF) of the lateral cerebral ventricle were performed in two of the goats via a permanently implanted three-cannula system as earlier described (Åkerlund Andersson and Olsson 1973) Isoprenaline hydrochloride dissolved in isotonic saline was infused into the ventricle at doses of 0 5 or 1 ng/kg min^{-1} In some experiments angiotensin II (Hypertensin Ciba) dissolved in 0 5 M NaCl, was infused into the CSF of the lateral ventricle subsequent to the intraventricular isoprenaline infusion. The dosage of angiotensin II was 0 8 ng/kg min^{-1}

Blood pressure and heart rate recordings Four of the goats had a polyvinyl catheter implanted via the superficial temporal artery into the carotid artery as described earlier (Eriksson Fernández and Olsson 1971) The free end of the catheter was attached to one horn of the goat and the catheter was flushed daily with heparin solution During the experiments the polyvinyl catheter was connected to a Statham pressure transducer and the systolic/diastolic blood pressures were recorded on an ink writing polygraph. When not registered on the polygraph the heart rate was determined at intervals by auscultation

Collection of saliva One of the goats was prepared with a polyvinyl catheter (O D 2 0 mm, I D 1.4 mm) ently implanted into the parotid duct (cf Hecker 1974) The free end of the catheter was passed through the skin of the cheek and then connected to a stainless steel barrel The barrel which was provided with an external and an internal flange was pushed through the skin opposite to the second premolar tooth In this manner the saliva was returned to the animal between experiments During experiments the free end of the catheter was disconnected from the barrel and the saliva was collected in graded glass tubes.

Hydration A water diuresis was established by giving the goats by stomach tube 100 ml kg b wt. of 38 C water 1½ to – h before the isoprenaline infusions were started

Analyses Urine was collected in 10 min samples via a retention catheter inserted into the urinary bladder Urine and salivary Na was determined by use of an EEL flame photometer and an "Advanced osmometer Inc." was used for determinations of the osmolality of these fluids and the blood plasma The mean plasma osmolality was found to be 290 mosm kg during hydration Therefore this value was used for calculations of renal free water clearance (C_{H_2O}) in the experiments performed in hydrated goats

Assay of antidiuretic hormone (ADH) in the urine The method described by Frandsen (1969) was employed with some modification for separation of ADH from urine and the amount of hormone present was assayed in the hydrated, nonanesthetized goat (Lisbajko 1975) This proceeding has been found to give approximately 10 per cent recovery in the urine of arginine vasopressin injected intravenously in the goat

Results

Intravenous infusions

Tests for effect on water intake 8 non hydrated goats were subjected to 60 min intravenous infusions of isoprenaline In 7 expts the low dose (0 1 μg/kg min $^{-1}$) was administered whereas the high dose (0 3 μg/kg min $^{-1}$) was given in 5 expts During these infusions the

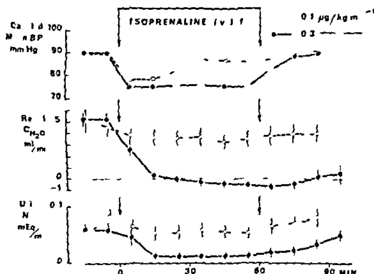


Fig. 1 Blood pressure and renal effects of two doses of isoprenaline hydrochloride infused intravenously in the conscious pre hydrated goat. Circles Moderate hypotension (n = 2) during an infusion of a low dose 0 1 μg/kg min⁻¹) of isoprenaline No obvious change in renal C_{H_2O} or in renal Na excretion (n = 7) Dots Marked hypotension (n = 4) antidiuresis and decreased renal Na^+ excretion during infusions of a high dose 0 3 μg/kg min) of isoprenaline (n = 7) Each symbol represents mean Vertical bars = S E

animals did not show any interest in the water available in front of them, but continued to eat hay now and again

Renal effects 6 pre hydrated goats were subjected to infusions of the *low dose* of isoprenaline (7 expts) As shown in Fig 1 (*circles*) these infusions did not cause any obvious change in the positive renal C_{H_2O} or in the renal Na excretion However when the *high dose* of isoprenaline was infused in 4 pre hydrated animals (7 expts) a marked drop in urine flow was observed within the first 5 min of the infusion However renal C_{H_2O} did not become negative until 30 min after the onset of the isoprenaline infusion (Fig 1 *dots*) Concomitant with the antidiuresis there was a marked fall in renal Na excretion In two of the expts the urine was collected before and during the entire periods of negative C_{H_2O} for separation of ADH Subsequent biological assay revealed the presence of roughly 5 m U of ADH in the urine collected during each of the periods of antidiuresis whereas no ADH was recovered in the preceding urine samples With an estimated recovery of 10 per cent it indicates that the 60 min intravenous infusion of the high dose of isoprenaline caused a release of approximately 50 mU of ADH

Effects on heart rate and arterial blood pressure All intravenous isoprenaline infusions induced tachycardia within few minutes During infusions of the low dose of isoprenaline the heart rate increased from about 90 beats/min to nearly 200 beats/min (Fig 2) whereas the heart rate was 200 to 250 beats/min during infusions of the high dose of the drug There was a moderate fall in blood pressure during the initial 20 min of the infusion of the low of isoprenaline Then the blood pressure gradually retained pre infusion val

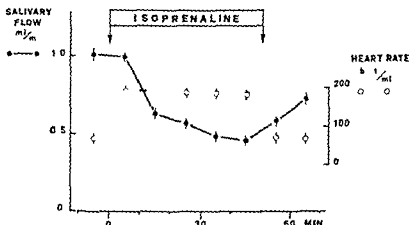


Fig 2 Marked decrease in the unilateral parotid salivary flow during intravenous infusions of isoprenaline at 0 1 μg/kg min⁻¹ Note the marked increase in heart rate Number of experiments 4 (mean and S E)

circles) A much more marked and sustained hypotension was observed during the intra venous infusions of the high isoprenaline dose (Fig 1 *dots*)

Effects on parotid salivary flow The secretion from one of the parotid glands was registered during 4 intravenous infusions of isoprenaline in one goat Isoprenaline was infused intra venously for 50 min at 0 1 μg/kg min⁻¹ Within 20 min of infusion the salivary flow had decreased from 1 1 ± 0 2 ml/min (mean and S E) to 0 6 ± 0 1 ml/min At the end of the in fusion period the flow rate was reduced to 0 5 ± 0 1 ml/min After cessation of the infusion the salivary flow slowly returned to pre infusion level (Fig 2)

Intraventricular infusions

Two non hydrated goats were used for infusions of isoprenaline into the CSF of the lateral ventricle The dose of isoprenaline was 0 5 ng/kg min⁻¹ (5 expts) and 1 ng/kg min⁻¹ (2 expts)

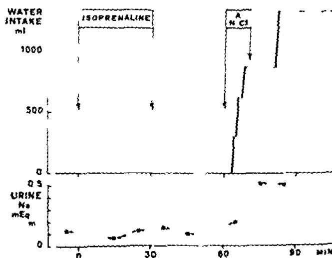


Fig 3 Lack of effects on water intake and renal sodium excretion during an isoprenaline infusion into the cerebrospinal fluid of the lateral ventricle Note the marked cumulative drinking and the natriuresis in response to the subsequent intraventricular infusion of angiotensin II dissolved in 0 5 M NaCl Dose of isoprenaline 1 ng kg min⁻¹ Dose of angiotensin 0 8 ng kg min⁻¹ Rate of infusion 0 7 l min

The infusion periods were 30 or 50 min None of these infusions induced drinking o affec e renal sodium excretion As expected (*cf* Andersson and Olsson 1973) however the anim responded to subsequent intraventricular infusions of angiotensin II in hypertonic NaCl w t cumulative drinking and natriuresis (Fig 3) Two 50 min intraventricular infusions of i o-prenaline (0 5 and 1 ng/kg min⁻¹) were also performed when the goats were hydrated The renal C_{H_2O} remained positive throughout these infusion periods

No tachycardia was observed during any of the intraventricular infusions of isoprenaline

Discussion

Systematically administered β adrenergic agonists act as very potent dipsogens in the water satiated rat (*cf* Falk and Tang 1974) and dog (Fitzsimons and Szczepanska Sadowska 1974) The present study shows that this is not a general phenomenon among mammalian species Infused intravenously in amounts previously shown to induce marked drinking in the dog isoprenaline did not elicit drinking in the goat The absence of dipsogenic response was apparently not due to a masking of thirst by sedative or nauseating effects of the drug since the animals frequently consumed hay during the infusions It can be concluded therefore that a particular effect of β adrenergic stimulation elicits an urge to drink in the rat and the dog but not in the goat As mentioned in the introduction evidence has been produced that the activation of the renin angiotensin system may be the ultimate cause of drinking occurring during β adrenergic stimulation in the rat (Houpt and Epstein 1971) Like rats (Epstein Fitzsimons and Rolls 1970) goats respond with drinking to the central application of angiotensin II (*cf* Andersson and Olsson 1973 and Fig 3) and it appears likely that the renin angiotensin system was stimulated to some extent during the isoprenaline infusions reported here However angiotensin II obviously was not liberated in amounts sufficient to stimulate the thirst mechanism

Isoprenaline induced drinking is apparently not due to an activation of the renin angiotensin system in the dog (Fitzsimons and Szczepanska Sadowska 1974) Furthermore recent studies have shown that the administration of angiotensin II blocking agents fails to attenuate β-adrenergically induced drinking in the rat (Tang and Falk 1974) It speaks against the idea that isoprenaline drinking is completely dependent upon the renin angiotensin system in this species Contribution of oropharyngeal factors of the kind responsible for normal prandial drinking in the rat cannot be excluded The dipsogenic response to isoprenaline is attenuated in surgically desalivated rats (Falk and Bryant 1973) Furthermore rats with lateral hypothalamic lesions continue to drink in response to isoprenaline (Lehr *et al* 1967) although such lesions are supposed to abolish both cellular and extracellular" (angiotensin induced) thirst leaving only the prandial drinking mechani m intact (Teitelbaum and Epstein 1962) Even the low dose of isoprenaline infused intravenously in the goat markedly reduced the flow of saliva (Fig 2) In contrast to rats and dogs (Wolf 1958) goats are not prandial drinkers and do not respond with drinking to atropine induced reduction of the salivary flow This seems to explain why the isoprenaline-evoked reduction in salivary flow did induce the goats to take water It also focuses the attention on the possibility t

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Acta physiol scand 1975 93 560–562
From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm, Sweden

Cyclic Excitability Changes of the Inspiratory 'Off-switch' Mechanism

By

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Previous results from this laboratory suggested that the combined vagal and central inspiratory activity projects to the inspiratory off switch mechanism and produces inspiratory termination when it has grown to a sufficient strength (Euler *et al* 1973) Results to be reported here have shed new light on these mechanisms

This study on cats is based on the findings of Cohen (1971) and of Bertrand and Hugelin (1971) that the inspiratory off switch mechanism can be activated directly also from a group of neurons within the medial parabrachial nucleus in the rostral pons in a time dependent manner We have stimulated this pool of neurons using brief tetanic stimulation s duration 0 2 s shock duration 0 5 ms at 300 Hz) The stimulus strength was adjusted to threshold for inspiratory inhibition The stimulus was triggered at varied delays from the onset of the inspiratory phrenic activity The cats were anesthetized with pentobarbital paralyzed by gallamine and ventilated according to its own demand by means of a servo-respirator driven by the integrated phrenic activity recorded from the C phrenic root The servo-respirator technique allowed the animal s own chemostatic control of ventilation to be maintained It also permitted us to switch off the respirator momentarily for one breath and thereby to record the inspiratory activity of an effort during which there was a complete absence of volume related feed back from the lungs

With this approach we have been able to test out the stimulus strength required to reach the threshold of the off switch neuron pool at various times from the onset of inspiration and thus to determine the time course of the declining inhibition of the off switch mechanism both in the presence and the absence of vagal afferent input and to determine the vagal contribution to the inspiratory off switch function The time course of the progressive fall in the threshold of the off switch mechanism was found to correspond closely to the time course of the growing inspiratory activity as it appears from the records of the integrated phrenic activity (*cf* upper and lower graphs of Fig 1) This suggests that the time course of the off-switch threshold whether determined as the volume threshold

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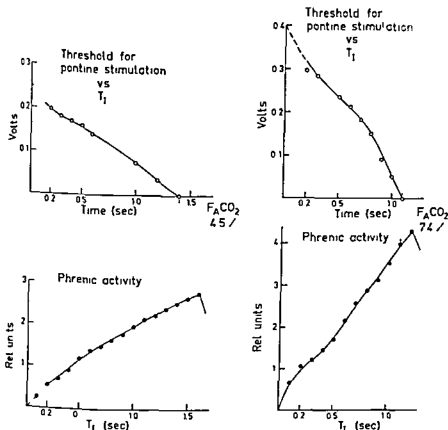


Fig. 1 Upper graphs show the time course of the falling threshold of the inspiratory off sw tch mecha sm without vagal feed back at two different end tidal CO levels The ordinate s gi en as inc ement above lowest hreshold Lower graphs show the t me course of the correspondi g integrated phren c activity

(Clark and Euler 1972 Bradley *et al* 1974 a b) or as the threshold to electrical stimulation of the parabrachial nucleus is dependent on and closely reflects the time course of the central inspiratory activity which projects also to the spinal inspiratory motoneuron pools (Euler *et al* 1973 *cf* Bradley *et al* 1975)

Another important result obtained with this technique is that the off switch threshold increases with increasing level of the chemical drive for ventilation This is illustrated in Fig 1 Bradley *et al* (1974 a b) have concluded on other grounds that the threshold is increased in response to an increase in inspired CO_2 concentration Our present experiments with electrical stimulation at the medial parabrachialis nucleus have now confirmed in a direct way these conclusions and shown that there are two basic primary responses of the respiratory control system to an increase in CO 1) an increased growth of the central inspiratory activity and 2) an increase in the threshold of the off switch neurons

These results provide an explanation also to the fact that in the absence of vagal feed back the inspiratory duration often stays relatively constant as the chemical drive increases

the increase in threshold often corresponds fairly closely to the simultaneously occurring increase in the rate of rise of central inspiratory activity so that their effects on the inspiratory duration largely cancel each other (Bradley *et al* 1975)

This work was supported by the Swedish Medical Research Council (Project no 14X 544) and by grants from Karolinska Institutets Fonder T T is indebted to the Royal Swedish Academy of Sciences for fellowship within the agreement for scientific exchange between the Polish Academy of Sciences and the Royal Swedish Academy of Sciences

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Acta physiol scand 1975 93 563-565
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Dopamine and Noradrenaline Content in the Brain of Rat Strains Selected for their Alcohol Intake

By

LIISA AHTEE and KALERVO ERIKSSON

Hereditary mechanisms are involved in the control of alcohol drinking in rats and mice (Mardones *et al* 1953 McLearn and Rodgers 1957) By outbreeding rats and selecting breeding animals that differ in their alcohol consumption Eriksson (1968 1969 1971) has developed 2 strains of rats one of which in a free-choice situation prefers a 10 (v/v) ethanol solution to water while the other drinks more water than ethanol solution Earlier we reported that the brain 5 hydroxytryptamine content is 15 to 20% higher in ethanol selecting than in water selecting rats (Ahtee and Eriksson 1972 1973) In the present work we have estimated the dopamine and noradrenaline content in the brain of the alcohol preferring and water preferring rats

The test animals were either ALKO Alcohol (AA) or ALKO Non Alcohol (ANA) male and female rats of Wistar origin that had been selectively outbred for their alcohol consumption for 22 generations These 2 rat strains have been described in detail by Eriksson (1969 1971) In the present experiments the AA rats were divided into 2 groups and the ANA rats into 3 groups (see Table I) During the free-choice experiments (treatment II) the rats were kept in individual cages Initially for a habituation period of 10 days they had 10% (v/v) ethanol solution as the sole drinking fluid After this time of habituation they were given a choice between water and 10 o ethanol solution In one series of experiments (treatment III) the ANA rats were forced to drink only ethanol as 10 solution The ethanol intake of the rats was measured daily The ethanol consumption is expressed as percentage of calories from ethanol in relation to total calorie intake The animals were killed by decapitation between 11 A M and 1 P M After decapitation the brains were rapidly removed dissected on ice and weighed Dopamine and noradrenaline were estimated in the same tissue sample by the method of Shellenberger and Gordon (1971) The results were corrected for the recovery of standard carried through the method The recovery was for dopamine 89 ± 1 5 (mean $\pm$S E 14 estimations) and for noradrenaline 86 ± 1 5 (14 estimations)

Table I shows that dopamine concentrations were 15 to 25 higher in the brain of

K y word Dopamine no adrenal ne ethanol preference

TABLE I The dopamine (DA) and noradrenaline (NA) content (μg/g mean ± S E.) in the brain of alcohol preferring (AA) and water preferring (ANA) rats before and during access to ethanol The table also gives percentage proportion (mean ± S D) of calories derived from ethanol day in relation to total calorie intake

Strain	Sex	Treatment[1]	Number of rats	Percentage of ethanol calories/day	DA	NA
ANA	♂	I	15	0	0 77 ± 0 02	0 40 ± 0 01
ANA	♂	II	8	6 ± 8 5	0 85 ± 0 02	0 44 ± 0 01
ANA	♂	III	10	49 ± 3	0 83 ± 0 0?	0 4 ± 0 00
AA	♂	I	10	0	1 05 ± 0 03[3]	0 45 ± 0 00
AA	♂	II	10	·7 ± 10	1 04 ± 0 0?[3]	0 47 ± 0 01
ANA	♀	I	4	0	0 77 ± 0 05	0 38 ± 0 01
ANA	♀	II	6	7 ± 5	0 79 ± 0 03	0 4 ± 0 01
AA	♀	I	9	0	0 97 ± 0 02[2]	0 41 ± 0 01
AA	♀	II	9	20 ± 15	0 93 ± 0 02[2]	0 41 ± 0 01

[1] I = Never ethanol II = Free choice of ethanol III = Forced ethanol drinking.
[2] Compared to corresponding ANA rats p < 0 01
[3] Compared to corresponding ANA rats p < 0 001

ethanol preferring rats than in the brain of water preferring rats The weight (mean ± S D = 1 54 ± 0 07 g) of the brain of the male ANA rats was 9% higher than that (1 41 ± 0 07 g) of the male AA rats Similarly the brains (1 48 ± 0 06 g) of female ANA rats weighed 11% more than those (1 33 ± 0 05 g) of the female AA rats However the dopamine concentrations were significantly higher in the brain of AA rats than in the brain of ANA rats if the total dopamine contents per brain rather than the concentrations were compared. Ethanol consumption either in a free-choice situation or in a forced situation did not change the brain dopamine concentration The brain noradrenaline concentrations of the AA rats did not differ significantly from those of the ANA rats As shown by Gordon and Shellenberger (1974) the noradrenaline concentration was slightly lower in the brain of female rats than in the brain of male rats

In conclusion these experiments show that the dopamine concentration but not the noradrenaline concentration is significantly higher in the brain of ethanol preferring rats than in the brain of water preferring rats Whether this difference bears any relation to the ethanol selecting behaviour of these rats remains to be elucidated

This study was supported by the Finnish Foundation for Alcohol Studies

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Acta physiol scand 1975 93 566–568

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Liver Function, Liver Hemodynamics and Intrahepatic Distribution of Portal Blood in Cats during Slight Hypothermia

By

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Previous experiments on cats have shown that a fall in body temperature to 36 C is accompanied by a pronounced depression in liver function (Larsen 1971 Krarup and Larsen 197?) It was suggested that the observed effect might be explained by restriction of the portal blood flow within the liver with a reduction of sinusoidal blood flow and functional capacity as a consequence The aim of the present study was to test this hypothesis by simultaneous registration of liver function and liver hemodynamics during cooling. Furthermore a series of experiments were carried out in which the effect of hypothermia on liver function was examined together with simultaneous rapid serial angiography of the hepatic portal vascular bed.

cats were fasted overnight and anesthetized with chloralose (50 mg kg) and an initial dose of Nem- (30 mg) In the control period the body temperature was kept at 38.5 C and th cat was cooled to ?6.0 C by blowi g an air stream over the chest. The operational technique flow measurements, estimation li er function analytical procedures and calculations have all been described elsewhere in detail (Krarup and Larsen 197_ Krarup 1973) The angiographic records were made in the entero-dorsal projection and the following contrast media were used in 19 series Isopaque _60 (Nyco Oslo) in 73 series Isopaque amine _80 (Nyco Oslo) and in 9 series Thorotrast (4-_f _ stabilized collodial thorium dioxide, Fell s Testagar Det o () The co trast med um was injected a body temperature in an amount of 0 6 ml 1s through a catheter (pol san tubing, I D 0 58 mm, O D 0 96 mm) placed in the portal vein or superior mesen eric ein I ectio press re was 4 kg cm² injection time I _ s. Examinations started simultaneous with the injection w th 3 exposures for 3 s followed by 1 exposure s for 10 s

In Table I is listed the m_ ked effect of cooling on liver function The splanchnic lact- output and the hepatic venous -hydroxybutyrate acetoacetate ratio was not affected indicating that there was no gross changes in the hepatic redox level. In the control period the portal venous and hep_) arterial resistance was 0 24 ± 0 05 and 9.2 ± 1.2 mm Hg ml kg min, respectively and did nange by cooling. The total liver blood flow was _4 ml kg b wt. min in the control pe iod with 29 ml passing through the portal vein and 15 ml through the hepatic artery Total blood flow and the distribution between the portal vein and the hepatic artery remained cons ant upon cooling.

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TABLE I The results from experiments in which the liver function was followed at normal to ture of 38 5 C and after cooling to a body temperature of 36 0 C

	Elimination rate of ethanol µmol/kg/min				Splanchnic blood flow ml/kg/min		Splanchnic oxygen uptake µmol/kg/min		ICG clearance ml/kg/min		ICG-extraction ratio per cent	
	Total		Splanchnic									
	A	B	A	B	A	B	A	B	A	B	A	B
Mean	38	29	31	21	51	49	124	99	6 3	4 7	18	13
E	1 6	2 7	3 6	3 2	7 1	6 0	19 4	19 3	0 7	0 5	2 3	1 7
6	p<0 005		p<0 005		p<0 3		p<0 005		p<0 001		p 0 01	

=control period B = cooling period kg = kg b wt

In the angiography experiments cooling of the cats caused a 30 per cent reduction in the elimination rate of ethanol but the fall in body temperature was not accompanied by any consistent changes in the angiograms In most of the series the angiograms showed what was considered to be normal angiograms of the liver i e there was uniform di tribution of the portal vein ramifications at the sinusoidal stage the contrast was evenly dispersed throughout the sinusoids the profile of the liver was smooth regular and well defined However in 12 out of 19 series with isopaque in 17 out of 71 series with isopaque amine and in 28 out of 92 series with thorotrast the angiograms revealed various degrees of restricted intrahepatic circulation as described by Daniel and Prichard (1951 a b) This was characterized by stunted appearance of the intrahepatic portal tree and the more peripheral segments of the vessels did not fill The sinusoidal shadow was confined to the areas immediately surrounding the stubby portal vessels with a patchy appearance of the shadow and the profile of the liver was irregular The appearance of restricted circulation was not related to changes in body temperature and did not influence the elimination curve of ethanol

The pre ent experiments have confirmed that the decrease in ethanol elimination rate caused by cooling is caused by a decrease in hepatic elimination rate The reduction in the hepatic elimination rate of ethanol corresponds to the fall in hepatic oxygen consumption and the results also correspond to previous experiments in which it was found that 36 per cent of the oxygen consumed by the liver was used for ethanol oxidation (Larsen and Krarup 1974) In accordance with the constancy of the hepatic hemodynamic parameters the experiments further demonstrate that cooling is not accompanied by gross changes in the hepatic portal vascular bed as visualized by angiography It is not possible to evaluate vessels with a diameter less than 0 5 mm However the diffuse opacity normally observed at the sinusoidal stage indicates that the contrast media are evenly dispersed throughout the sinu oids The experiments have confirmed the existence of restricted intrahepatic circulation as demonstrated by Daniel and Prichard (1951 a b) They offered no explanation for these vascular changes and these experiments exclude that slight changes in body temperature are responsible for the observed restriction of blood flow The results also demons that restrictive flow is not accompanied by functional changes in the liver as juc

the elimination rate of ethanol This is in accordance with the incidental and non reproduceable character of the restriction Most probably the vascular changes are transient and of short duration caused by specific properties of the contrast media and/or unspecific properties such as osmolarity p_H and cation content This would be in accordance with similar findings in nephroangiography (Edsman 1957 Sherwood and Lavender 1969)

In conclusion the experiments have demonstrated that the depressive effect of slight cooling on liver function is not related to gross changes of the intrahepatic vasculature as judged from hemodynamic and angiographic parameters The occasional occurrence of restricted intrahepatic portal circulation observed by angiography appears to be of transient nature without measurable effect on liver function and is most probably caused by a vascular reaction to the contrast media

The technical assistance of Miss S Holm Mrs J Justesen and Mrs L Nielsen is gratefully acknowledged

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Acta physiol scand 1975 93 569–571
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Rate of Energy Utilization in the Cerebral Cortex of Rats

By

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There are few methods available for the measurement of metabolic rate in brain tissue and apart from a recently described technique for autoradiographic estimation of glucose phosphorylation (Reivich 1974) only one method allows regional studies This method which was described by Lowry *et al* (1964) is based on the assumption that when the cerebral circulation is interrupted the metabolic rate is initially maintained at pre-ischemic values Since the oxygen stores of the tissue are virtually nil energy can only be made available from the existing stores of high-energy compounds and from glycolytic production of ATP Accordingly the rate of energy ($\sim$P) utilization can be calculated from the equation $\Delta\sim P = 2\Delta ATP + \Delta PCr + \Delta ADP + 2\Delta Glucose + 2.9\Delta Glycogen$ where ΔPCr denotes the change in phosphocreatine concentration 2.9 is a figure derived on the assumption of a 10 % branching of glycogen (see Lowry *et al* 1964) If glycogen is not measured $\sim$P production from glycolysis can be obtained from changes in glucose and lactate (Gatfield *et al* 1966)

One of the difficulties with the closed box method is that since following decapitation $\sim$P utilization falls continuously the period of ischemia must be accurately assessed and the tissue must be frozen instantaneously Furthermore the validity of the method has never been tested by comparisons with other estimates of metabolic rate The present experiments were undertaken to measure $\sim$P utilization in nitrous oxide and phenobarbitone anesthesia after 5 10 and 20 s of ischemia using rapid freezing of cortical tissue in rats The values obtained were compared with those derived from measurements of cerebral metabolic rate for oxygen (CMR_{O_2})

Groups of unstarved Wistar rats (300–400 g) were initially anesthetized with 2–3% halothane and subsequently maintained artificially ventilated on 70 % N_2O Other groups were anesthetized with phenobarbitone (150 mg/kg) Body temperature was kept close to 37 C and arterial P_{CO_2} and P_{O_2} were adjusted to 35–40 and 120–140 mm Hg, respectively A craniectomy on one side (about 10 15 mm) exposed the dura over the fronto-parietal cortex The tissue was frozen either without or with previous interruption of cerebral circulation by decapitation using isopenthane at −160 C which was allowed to flow across the exposed dura The periods of ischemia, *i e* the time between decapitation and freezing of the tissue were either 5 10 or 20 s (N_2O anesthesia) or 10 and 20 s (phenobarbitone anesthesia) Control experiments showed that the superficial 1 mm of the cerebral cortex fr

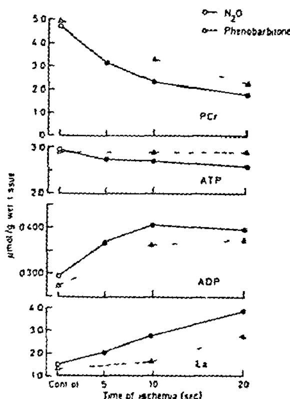


Fig. 1 Rate of changes in cerebral cortex concentrations of phosphocreatine (PCr) ATP ADP and lactate following decapitation in rats under N_2O or phenobarbitone anaesthesia Filled symbols denote significant changes from control values ($p < 0.05$) S E values not given since they were smaller than the size of the symbols

in 1–2 s and this part of the tissue was therefore used for analyses The tissue was dissected and extracted at −22 C and PCr ATP ADP and lactate were determined using enzymatic fluorometric techniques (see Folbergrová *et al* 1972) Since control experiments showed that there was no significant decrease in glycogen in the periods studied ~P utilization was calculated from $\Delta{\sim}P = 2\Delta ATP + \Delta ADP + \Delta PCr + \Delta lactate$

Fig. 1 illustrates the changes in PCr ATP ADP and lactate in animals anesthetized with either N_2O or phenobarbitone The rate of fall in PCr and ATP and the increase in lactate content were clearly lower during phenobarbitone anesthesia Table I shows the ~P values calculated for the ischemic periods studied and compares the values to those derived from

TABLE I Energy utilization in the rat cerebral cortex calculated from closed box and from CMR_{O_2} measurements

Anesthesia	Number of animals	Time after decapitation (sec)	~P utilization (µmol g⁻¹ min)	Energy flux (~P) calculated from CMR_{O_2} (µmol g⁻¹ min)
N_2O	8	5	30 ±	
	8	10	4	[illegible]
	4	20	17 ± 1	
Phenobarbitone	4	10	11 ±	
	3	0	1 ± 1	15

measurements of CMR_o in nitrous oxide anesthesia (Norberg and Siesjö 1974) or in animals given phenobarbitone 150 mg/kg (Nilsson and Siesjo 1975) CMR_o was converted to ~P utilization by assuming that 1 μmol of glucose is completely oxidized with an energy yield of 38 μmol ATP

The values obtained in the nitrous oxide group demonstrate that the method of Lowry *et al* (1964) gives values for energy flux that are continuously decreasing within 20 s after decapitation However it turns out that in these lightly anesthetized animals the ~P utilization as calculated after a 5 s period of ischemia corresponds to the value derived from CMR_o, On the other hand in phenobarbitone anesthesia the calculated energy flux remains constant and close to the measured in vivo flux at least up to 20 s of ischemia The reason for this is not known but may signify that ~P utilization is maintained close to the normal pre ischemic values only as long as there is a near normal ATP concentration Thus the method can only be used in lightly anesthetized animals if very short ischemic periods are employed On the whole though the present results demonstrate that the closed box method of Lowry can be a valuable tool for studies of regional metabolic rates

This study was supported by grants f om the Swedish Med cal Research Council (Projects No 14x 179 and 14x 263) from the Bank of Sweden Terce itenary Foundation and by U S PHS Grant No 5 R01 NS 07838 05 from NIH

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Acta physiol scand 1975 93 572–573

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Evidence for an α- and β_2-Receptor Mediated Inhibition of the Twitch Response in the Guinea Pig Vas Deferens by Noradrenaline

By

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In their studies on the nature of the motor transmission in the guinea pig vas deferens and the effects of noradrenaline (NA) on the twitch, Ambache and Zar (1971) and Ambache *et al* (1972) have reported that phenoxybenzamine leaves the inhibitory effect of NA on the twitch unaffected or unmasks it whereas it is annulled by phentolamine They have also denied any β-adrenergic contribution to the NA induced inhibition of the twitch.

In the present report we wish to present some evidence for an inhibitory action of exogenous and endogenous NA on the motor transmission in the guinea pig vas apparently mediated both by α and β_2-receptors

Guinea pig vasa deferentia were mounted in a 5 ml bath with Tyrode as bath fluid (for composition see Hedqvist and Euler 1972) gassed with 95% O_2 and 5% CO_2 and kept at 37 C Transmural stimuli were delivered by a Grass S4 stimulator through platinum wires along the wall of the bath Twitches were elicited at 1 min intervals by 8–25 biphasic pulses at 4–10 Hz, pulse duration 1 ms supramaximal voltage The contractions were recorded by an isotonic transducer on a Honeywell ink writer The load of the organ was 0.25 g. The following drugs were used Butoxamine hydrochloride clonidine hydrochloride (−)noradrenaline bitartrate phenethylamine hydrochloride phenoxybenzamine hydrochloride phentolamine hydrochloride practolol propranolol chloride terbutaline sulphate tyramine hydrochloride

In concentrations of 0 1–1 μg/ml NA caused either inhibition or enhancement of the twitch or a combination of both After addition of phenoxybenzamine or phentolamine (1 μg/ml) the effect of NA was invariably an inhibition of the twitch The NA induced inhibition remaining after treatment with an α blocker was now annulled by propranolol or by butoxamine (2 μg/ml) but not by practolol suggesting that the inhibition left after α blockade was mediated by β_2-receptors (Fig. 1)

In order to study the effect of endogenously released NA we have examined the effect of tyramine (TA) or phenethylamine (0 5–5 μg/ml) on the twitch These drugs produced the same kind of effects on the twitch as exogenous NA In some cases phentolamine abolished the TA induced inhibition as noted by Ambache *et al* (1972) with another α blocker phenoxybenzamine In other cases an inhibition remained after phentolamine which was then annulled by butoxamine (Fig. 2)

An inhibitory effect on motor transmission of the twitch can also be demonstrated with

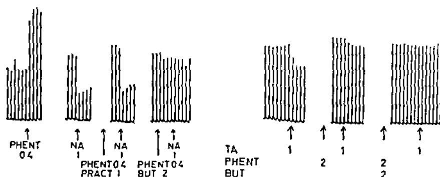


Fig 1 Twitch responses to transmural stimulation (10 Hz ₄5 pulses 1 ms supramaximal voltage) at 1 min intervals in guinea pig vas deferens Effect of NA after respectively phentolamine phentolamine + practolol, and phentolamine + butoxamine All doses in μg/ml

Fig 2 Twitch responses to transmural stimulation (5 Hz 10 pulses, 1 ms supramaximal voltage) at 1 min intervals in guinea pig vas deferens Effect of TA before and after respectively phentolamine and phentolamine + butoxamine All doses in μg/ml

the α agonist clonidine (0 02–0 1 μg/ml) This inhibition is prevented or abolished by phentolamine 0 02–0 1 μg/ml The β agonist terbutaline (0 1–0 2 μg/ml) also inhibits the twitch an effect which is antagonized by butoxamine (2 μg/ml)

From these results we conclude that NA either added to the isolated vas or released endogenously from the stores may inhibit the twitch by an action on α as well as β_2 receptors In their study Ambache and Zar (1971) found that phentolamine blocked the NA induced inhibition in the presence of propranolol Our experiments have shown that propranolol (or butoxamine) abolish the inhibition in the presence of phentolamine

Since even prolonged exposure of the vas to the non-competitive α blocker phenoxy benzamine in combination with propranolol does not conspicuously alter the twitch we conclude in agreement with Ambache and Zar (1971) that the motor transmission of the twitch is non adrenergic It seems conceivable that the increase in the twitch observed after α blockers particularly phentolamine is due to removal of an inhibition possibly caused by small amounts of NA released during stimulation especially since this effect is more marked at pulse trains of 2–5 s than of 0 5 s duration

In summary our findings support the conclusion reached by Ambache and Zar (1971) that the motor transmission of the twitch in the guinea pig vas deferens is non adrenergic and that the effect of NA on the transmission is inhibitory In addition our experiments indicate that the inhibition of transmission induced by exogenous or endogenous NA may be mediated both by α and β_2 receptors presumably located pre junctionally

This study was supported by grants from the Swedish Medical Research Council project no 04X-434

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Acta physiol scand 1975 93 574–576

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Catecholamine Content in the Interscapular Adipose Tissue and Adrenal Gland of Cold-Acclimatized Guinea-Pigs

By

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Nonshivering thermogenesis is associated with a marked rise in catecholamine release primarily of noradrenalin from adrenergic nerve terminals but also of adrenalin from the adrenal medulla (Himms Hagen 1965)

Particularly the action of catecholamines on brown adipose tissue has received much attention in recent years However the biochemical basis for the cold induced increase in metabolic heat production in larger mammals as in the man which do not normally possess considerable amounts of brown adipose tissue is poorly understood

For cold exposure studies we have searched for a good laboratory animal model which in its responses would resemble man as much as possible The guinea pig seems to come much closer to man when regarding the structure and enzymehistochemical pattern of interscapular adipose tissue (IAT) than the rat In the human brown fat is rarely seen in adults (Hassi 1971) and the reactions of oxidative enzymes disappear from the fat cells on aging as occurs also in the guinea pig (Hassi unpublished observations Hirvonen 1969)

In previous experiments it was found that histochemical reaction of monoamine oxidase demonstrable in the interscapular fat cells of guinea pigs acclimatized to cold (H t nen et al 1973) The phenomenon speaks in favor of enhanced breakdown of monoamines in the fat cells It also indirectly suggests that more monoamines are transmitted to the fat cells

In the present work the hypothesis was tested whether the adrenergic innervation of the adipose tissue of guinea pigs gets stronger during cold acclimatization The adrenal glands were also analysed for their catecholamine content in order to see whether the acclimatiza tion is also associated with increased amount of adrenal amines

Eight adult male guinea pigs were exposed to cold at +4 C for months The other 8 served as contr ls and they were kept at room temprrature (+ 0 C) Both groups were given similar food ad l bitum The animals were killed by a blow on the head and IAT as well as adrenal glands were excised

The catecholamine fluorescence in the adrenergic nerves in the IAT and in the adrenal medulla was visualized w th the paraformaldehyde vapor technique (Eränkö 1967) which was slightly mod fied Sections of 15 μm were cut in a cryostat (at 5 C) and freeze dried in an exsiccator over phosphorus pentoxide at − 0 C for 1 h The dry sections were placed in a jar with paraformaldehyde to 80 C for 1 h The sec tions were viewed and photographed in a fluorescence microscope (Leitz) The excitation f lter was LG 1 and barrier filter K 430 (Schott) Xylene was used as the mounting medium The nerve endings fluoresced with deep blue colour Becaus of the great intensity of the fluorescence the colour was yellow h blue in the adrenal medulla Noradrenalin content of the IAT was measured according to Shore and Ol n (1958) and adrenalin of the adrenal gland by the mod fied method of Shore and Olin (Vapaatalo 1968)

Key words Catecholam nes brown adipose tissue cold acclimatization



1



2

Fig 1 Interscapular adipose tissue of a cold acclimatized guinea p g No adrenalin fluorescence is seen in the w ll of arterioles and between the fat cells 160 Formaldehyde vapor treatment

Fig Interscapular adipose tissue of a control non acclimatized gu nea pig Noradrenaline fluorescence is seen in the arterial wall No fluorescence has been de eloped between the fat cells

There was more intense fluorescence in the walls of the arteries and arterioles of IAT in cold acclimatized animals than in the controls Fluorescence was also increasingly accu mulated in the immediate vicinity of the fat cells which were of the multilocular type The adrenergic innervation of IAT in the control guinea pigs was scarce and restricted to the arterial walls The fat cells were of the unilocular type (Figs 1 and 2)

There was no noticeable difference between the groups in respect of the amount of the fluorescence in the adrenal medullas

The mean noradrenalin content of the IAT in the cold acclimatized guinea pigs was about 60 higher ($p < 0\ 05$) than in the controls (1 62 μg/g versus 1 01 μg/g Table I) The average concentration of adrenalin in the adrenals was the same in both groups

Whether only the amount of the transmitter was increased or the nerve endings had grown more abundant could not be settled The small nerve endings which appeared between the fat cells seemed similar to those fibres resistant to surgical and immunosympathectomy found in the brown fat of the rat (Derry *et al* 1969)

The working hypothesis derived from the monoamine oxidase reaction was supported by the results at least the amount of noradrenalin was increased in the IAT and at the fat cells The measurements of amine concentrations coincided well with the histochemical

TABLE I The concentration of catecholamines in the interscapular adipose tissue (IAT) and in the adrenal gland of guinea pigs

Guinea pigs	IAT μg/g wet tissue mean ± S D	Adrenal gland μg/mg wet tissue mean ± S D
Cold acclimatized (N = 8)	1 61 ± 0 65	0 27 ± 0 04
Controls (N = 8)	1 01 ± 0 53	0 27 ± 0 07

observations We are unaware of similar experiments on guinea pigs but the results are parallel to those on the brown fat of cold acclimatized rats (Cottle *et al* 1967 Cottle and Cottle 1970)

No change in the amount of catecholamines in the adrenals of the cold acclimatized guinea pigs was noticed This does not, however exclude the possibility that the turnover of catecholamines were increased due to cold acclimatization

It is an empirical fact that man is also acclimatized to cold but the physiological and metabolic mechanisms are not well known We have found (unpublished results) in necropsies that brown fat is retained around mediastinal vessels in persons who are outdoor workers (lumberjacks carpenters) Guinea pigs reared in cold also retain their interscapular brown fat (Bruck and Wunnenberg 1965) This has led to the hypothesis that the improved ability to withstand cold in persons working outdoors is at least partly due to retaining more brown fat or even to increasing its amount as seen in guinea pigs

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